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Review

Molecular requirements of lignin–carbohydrate complexes for expression of unique biological activities

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Abstract

Lignins are major cell wall components formed by the dehydrogenative polymerization of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols. We prepared lignin–carbohydrate complexes (Fr. VI and Fr. VII) from pine cones by acid and ethanol precipitation, and investigated which part of these molecules is essential for expression of biological activities. They showed potent antiviral activity upon direct interaction with the virus. The antiviral activity of Frs. VI and VII required the higher-order structure of polyphenols without polysaccharides. Pretreatment of mice with Fr. VI or VII induced higher antiparasite activity than those of natural and chemically modified antitumor polysaccharides. Fr. VI or VII at higher concentrations enhanced the radical intensity and cytotoxic activity of vitamin C, whereas tannins counteracted the effect of vitamin C. Fr. VI at lower concentrations enhanced the O_2^--scavenging activity of vitamin C. Frs. VI and VII stimulated mouse macrophage-like cells Raw 264.7 to produce nitric oxide (NO), citrulline (CIT) and asparagine (ASN), via the enhanced expression of iNOS and ASN synthetase, whereas phenylpropenoid monomers and polymers inhibited NO/CIT/ASN production. These data suggest that the polymerized structure of phenylpropenoids in lignin–carbohydrate complexes is required for the induction of antiviral activity, and that the higher-order structure of phenylpropenoid polymers and polysaccharides is required for immunopotentiation, including macrophage activation.

Keywords: *Pinus parviflora* Sieb. et Zucc.; Pinaceae; Pine cone; Lignin; Polyphenol; Antiviral activity; Antitumor activity; Macrophage; Nitric oxide; Asparagine; iNOS

Contents

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1. Introduction

Lignins are a major class of natural product constituents in the land-plant kingdom, and are formed through phenolic oxidative coupling processes (Davin et al., 1997). Lignin macromolecules are formed by the dehydrogenative polymerization of three monolignols: *E*-*p*-coumaryl, *E*-*p*-coniferyl and *E*-sinapyl alcohols (Lewis and Yamamoto, 1990). Some polysaccharides in the cell walls of lignified plants are linked to lignin to form lignin–carbohydrate complexes, and the lignin–carbohydrate complexes from bald cypress, birch and rice straw show an extremely broad molecular weight distribution, from $1.5–8.5 \times 10^5$ and $1.5–10.0 \times 10^3$ (Azuma and Koshijima, 1988). In their role as integral cell wall components, lignins help provide mechanical support and defend against pathogens and herbivores. However, very little attention has been paid to the biological activities of lignins or lignin-containing/derived substances. Therefore, we investigated the diverse biological activities of lignin–carbohydrate complexes, such as anticancer, antioxidant, prooxidant, apoptosis-inducing, antimicrobial, antiparasite and anti-HIV activities. Due to the molecular complexity of lignin–carbohydrate complexes, it has been difficult to assign these biological activities to specific structural components, compared to the activities of chemically defined tannins and flavonoids (Okuda et al., 1991). To achieve such assignments, we prepared natural lignin–carbohydrate complexes (Fr. VI and Fr. VII), mostly from pine cones of *Pinus parviflora* Sieb. et Zucc., those without a sugar moiety by treatment with acid, those without phenylpropenoid polymers by digestion with $NaClO_2$, and dehydrogenation polymers of phenylpropenoids such as ferulic acid, caffeic acid and *p*-coumaric acid. Using these preparations, we investigated which part of the lignin–carbohydrate complex structure is responsible for these biological activities. In some cases, we compared the biological activities of Fr. VI and Fr. VII with those of commercially available lignins and chemically defined hydrolyzable tannins, which have a cyclitol in the center, attached by an ester bond to gallic acids, a hexahydroxyldiphenoyl (HHDP) group (dimer of gallic acid) or valoneoyl group (trimer of gallic acid) (Okuda et al., 1991), and condensed tannins, which have flavan units (mainly (+)-catechin, (−)-epicatechin or their derivatives) condensed with each other by a C–C bond.

2. Results and discussion

2.1. Identification of lignin as an antitumor component of pine cone extract

Lignin–carbohydrate complexes were isolated from hot water and alkaline extracts of pine cones of *P. parviflora* Sieb. et Zucc. based on folklore that the hot water extract is effective for treating gastroenterological tumors. Since there is no scientific documentation on this point, we isolated several bioactive substances from this extract. We first isolated differentiation-inducing substances, which induced the differentiation of mouse myeloid leukemia M1 cells into macrophage-like cells with higher NBT-reducing activity and non-specific esterase activity. The differentiation-inducing substance was a large molecule (as judged by elution at the void volume of Sephadex G-200) (Sakagami et al., 1986) and acidic (as judged by tight binding to a DEAE–Sepharose CL-6B column and elution by 0.15 M NaOH after washing with 1 M NaCl) (Ikeda et al., 1988a). We next isolated antitumor substances. Activity that significantly prolonged the survival of mice that had been implanted with ascites sarcoma-180 cells in the peritoneal cavity was recovered from both hot water and alkaline extracts, after delipidation of pine cone with ethanol. The activity in the hot water extract was tightly bound to a DEAE–cellulose column, and the fraction eluted with 0.15 M NaOH after washing with 2 M NaCl was referred to as Fr. V. Most of the activity in the alkaline extract was precipitated by acidification (pH 5) (this fraction is referred to as Fr. VI), and also by ethanol (50%) precipitation of the post-Fr. VI supernatant (this fraction is referred to as Fr. VII). These fractions effectively reduced the tumor volume of subcutaneously implanted sarcoma-180 cells (Sakagami et al., 1987). These fractions were dialyzed against water and were subjected to chemical analysis. As assessed by Sepharose CL-4B gel filtration

chromatography, the molecular masses of Frs. V and VI were estimated to be 10–70 and 70–200 kDa, respectively. Elemental analysis demonstrated the presence of C, H and O. Gas chromatography showed the presence of neutral sugar (arabinose (or fucose), mannose, galactose and glucose) (11–20% of dried weight) and uronic acid (1.7–5.2%) in Fr. VI and Fr. VII (Sakagami et al., 1987).

The following chemical analysis allowed us to identify Fr. VI as lignin–carbohydrate complexes. (a) UV absorbance spectrum: minimum absorption at 260 nm, maximum absorption at 280 nm, broad maximum absorption at 500 nm, and endoabsorption up to visible region, (b) IR spectrum: hydroxyl group with hydrogen bonding (3400 cm^{-1}), aliphatic C–H (2700 cm^{-1}), carbonyl group conjugated to π-electron system (1700–1600 cm^{-1}), aromatic double bond (1600, 1500 cm^{-1}), C–O expansion and contraction (1400–1000 cm^{-1}), no ester bonding. (c) ESR spectrum: one strong signal at $g = 2.003$ in the solid state at room temperature. The signal intensity was significantly reduced by oxidation and reduction. (d) ^1H NMR spectrum: When measured in 0.2%NaOD–D_2O, the spectrum suggested the presence of hydrogens in aromatic CH (δ 6.5–7.5 ppm), >C=C< (δ 4.5–5.5 ppm) and –O–CH–CH (δ 3.0–4.0 ppm). When the sample was acetylated with pyridine–acetic acid anhydride and dissolved in $CDCl_3$, the presence of acetyl groups bound to phenolic OH (δ 2.3 ppm) or to alcoholic OH (δ 2.1 ppm) moieties was confirmed. (e) Cellulose thin layer chromatography: The R_f value of Fr. VI was the same as that of commercial alkali–lignin in various solvent systems (10:0, 8:2, 5:5, 2:8 or 0:10 mixture of 1 N HCl and dioxan) (Sakagami et al., 1989).

Lignin–carbohydrate complexes (Frs. VI and VII) prepared by acid and ethanol precipitation from alkaline extracts of pine cones of *P. parviflora* Sieb. et Zucc, *Pinus densiflora* Sieb. et Zucc., *Pinus thunbergii Parl.*, *Pinus elliottii* var. Elliottii, *Pinus taeda* L., *Pinus caribaea* var. Hondurenses, *Pinus sylvestris* L., and even the pine seed shells of *P. parviflora* Sieb. et Zucc. and *Pinus armandii* Franch. induced comparable antitumor activity in mice. However, the yield of lignin–carbohydrate complexes was the highest when pine cones of *P. parviflora* Sieb. et Zucc were used as a starting material (Harada et al., 1988).

2.2. Induction of antimicrobial and antiparasite activity by lignin

Pretreatment of mice with Fr. VI or VII, prepared from pine cone and seed shell from various sources as described above, induced antimicrobial activity against various microorganisms (*Staphylococcus aureus* SH10, *Escherichia coli* GN2411, *Pseudomonas aeruginosa* H7, *Klebsiella pneumoniae* ST101, *Candida albicans* YA2), but not *Salmonella enteritidis* 116-54 (Harada et al., 1988; Oh-hara et al., 1990). When the sugar moiety of Fr. VI was destroyed by treatment with sulfuric acid or trifluoroacetic acid, the antimicrobial activity was significantly reduced. Furthermore, dehydrogenation polymers of phenylpropanoids, which do not contain sugar, showed much lower antimicrobial activity. Injection of Fr. VI or VII by any route (*s.c.*, *i.p.*, *p.o.*) induced higher antiparasite activity than other natural antitumor polysaccharides (PSK, Schizopyllan, TAK, carboxymethyl TAK) (Abe et al., 1989) (Table 1). These data suggest that the conjugation of sugar and polyphenol is essential for the expression of in vivo biological activity. Injection of Fr. VI or VII into the peritoneal cavity induced the accumulation of macrophage, and enhanced the production of active oxygen (measured by luminol chemiluminescence), which is highly toxic to tumor cells and bacteria (Harada et al., 1988).

2.3. Induction of endogenous cytokines

When mice were pretreated intravenously with Fr. VI or Fr. VII, the production of cytotoxic factor elicited by OK-432 (Picibanil) (Sakagami et al., 1990a) or by *Lactobacillus casei* (Sakagami et al., 1992b) was further enhanced, accompanied by the accumulation of Kupffer cells in the liver. The enhanced production of cytokine might be involved in the induction of antitumor, antimicrobial and antiparasite activity by lignin–carbohydrate complexes. In fact, endogenously produced cytokines such as TNF have been reported to induce resistance against microbial infection (Nakane et al., 1988; Shemer-Avni et al., 1988). The cytokine production (TNF-like cytotoxic factor) stimulated by lignin–carbohydrate complexes decreased during aging of mice and upon tumor implantation (Hanaoka et al., 1989).

Table 1
Pine cone lignin–carbohydrate complexes (Frs. VI, VII) induced antiparasite activity, while other antitumor polysaccharides did not

Treatment	Route	Age of mice	Inhibition (%)
Fr. VI	*s.c.*	1	92
		4	0
	i.p.	1	39
		4	82
	p.o	1	67
		4	87
Fr. VII	*s.c*	1	72
		4	0
	i.p	1	30
		4	92
	p.o	1	16
		4	86
PSK	*s.c*	1	0
Schizophyllan	*s.c*	1	0
TAK	*s.c*	1	5
Carboxymethyl TAK	*s.c*	1	4

2.4. Pharmadocynamics

To investigate the distribution of lignin–carbohydrate complexes in various organs and tissues, we prepared radioactive Fr. VI by mixing it with $Na^{125}I$. The mixture was dialyzed against distilled water to remove free $Na^{125}I$ and lyophilized (Sakagami et al., 1992c). When [^{125}I]Fr. VI was intravenously injected, significant radioactivity [about 3–8% corrected after recovery (32–60%) from animals] was accumulated in the liver, and thereafter excreted into urine and feces. These results indicate that some Fr. VI can reach the target organs after being absorbed (Sakagami et al., 1999a). Intravenously administered Fr. VI maintained its anti-HIV activity for a certain period of time (Shimizu et al., 1993).

The safety and antimutagenic activities of Frs. VI and VII were confirmed by Ames' method (Lee et al., 1993; Asanoma et al., 1993). Fr. VI inhibited the metabolic activation of benzo[*a*]pyrene. The antimutagenic activity of lignin–carbohydrate complexes may be due to inhibition of the generation of promutagens or mutagens, or by interaction with these substances (Lee et al., 1993). Recently, lignins have been reported to inhibit *N*-methyl-*N*′-nitro-nitrosoguanidine (MNNG)-induced DNA strand breaks and gene mutation in vitro (Labaj et al., 2003). This antimutagenic activity of lignin against MNNG may result from adsorptive and antioxidative action (Labaj et al., 2003) or scavenging activity against reactive oxygen species (ROS) (Ebringer et al., 2003).

When Fr. VI was injected intravenously, hemooxygenase (which is a rate-limiting enzyme in heme degradation) was activated, whereas cytochrome P-450, aminopyrine *N*-demethylase and aniline hydroxylase activities were slightly inhibited (Sakagami et al., 1999a). Since promutagens are bioactivated by cytochrome P-450, inhibition of this enzyme by Fr. VI may explain its antimutagenic activity. The cytochrome P-450 (CYP) family and transcription factors, the expression of which is modified by lignin, need to be identified.

2.5. Antiviral activity

Frs. VI and VII inhibited the multiplication of human immunodeficiency virus (HIV) in the cells (Lai et al., 1990). Anti-HIV activity was assessed by a selectivity index ($SI = CC_{50}/EC_{50}$, where CC_{50} is the 50% cytotoxic concentration against mock-infected cells, and EC_{50} is the 50% effective concentration against HIV-infected cells). The anti-HIV activity of Frs. VI and VII (SI = 4–124) was significantly higher than that of tetrameric ($SI = 7.3 \pm 6.5$, $n = 3$), trimeric ($SI = 3.4 \pm 3.7$, $n = 4$), dimeric ($SI = 2.3 \pm 3.2$, $n = 39$) and monomeric hydrolyzable tannins ($SI = 1.8 \pm 2.8$, $n = 21$), and condensed tannins ($SI = 1.1 \pm 0.4$, $n = 8$) (Nakashima et al., 1992b). The anti-HIV activity of lignin–carbohydrate complexes was not affected by treatment with acid, which removes monosaccharide residues through acid-catalyzed hydrolysis, but was significantly reduced by $NaClO_2$, which greatly destroys the lignin structure. Glucans, polymers of glucose, were inactive. Furthermore, phenylpropanoid polymers showed higher anti-HIV activity (SI = 105–178) than natural lignin–carbohydrate complexes. These data demonstrate that anti-HIV activity is mostly derived from the polyphenol portion of lignin–carbohydrate complexes. Since phenylpropenoid monomer was inactive ($SI < 1$), the higher-order structure of polymerized phenylpropenoids is essential for the expression of anti-HIV activity (Lai et al., 1992). The anti-HIV activity of lignin–carbohydrate complexes may be due to the inhibition of HIV adsorption to target cells (Nakashima et al., 1992a) or to the inhibition of reverse transcriptase, a replication enzyme of HIV (Lai et al., 1990).

We have reported that lignin–carbohydrate complex fractions (Frs. VI, VII) of Catuaba bark (Manabe et al., 1992), pine seed shell (Sakagami et al., 1992d), *Acer nikoense* Maxim. (Sakagami et al., 1997a), *Ceriops decandra* (Griff.) (Sakagami et al., 1998) and *Crataegus Cuneata* Sieb. et Zucc. (Satoh et al., 1998) showed anti-HIV activity that was comparable to that of the corresponding fractions of pine cone. Crude alkaline extract of pine cone of *P. elliottii* var. Elliottii showed slightly weak, but significant, anti-HIV activity, compared with purified lignin–carbohydrate complexes. Further purification of pine cone lignin–carbohydrate complex fraction (Fr. VI) by gel filtration did not significantly enhance anti-herpes simplex virus activity (Fukuchi et al., 1989a). For the commercialization of active substances, both the activity and the yield should be considered (Satoh et al., 1999b).

Although the antiviral activity of lignins was much lower than those of other popular anti-HIV agents such as sulfated polysaccharide (paramylon sulfate, dextran sulfate, curdlan sulfate, pentosan polysulfate) (SI = >563, >1130, >1748, >819) (Koizumi et al., 1993), 3′-azido-2′,3′-dideoxythymidine (AZT) (SI = 6609) (Nakashima et al., 1994) and dideoxycytidine (ddC) (SI = 2283) (Motohashi et al., 2002), lignins showed relatively broad antiviral spectra.

Lignin–carbohydrate complexes (Frs. VI and VII) inhibited plaque formation by the influenza virus in vitro, as well as RNA polymerase, a replication enzyme of influenza virus (Nagata et al., 1990). A limited digestion experiment demonstrated that the anti-influenza virus activity of Frs. VI and VII is derived from the polyphenol portion, not from the polysaccharide portion (Harada et al., 1991). This was substantiated by the observation that phenylpropanoid polymers showed anti-influenza virus activity that was comparable to or higher than that of natural lignin–carbohydrate

complexes (Sakagami et al., 1990b). When influenza virus was mixed with lignin–carbohydrate complexes, the lethal effect of the intracerebral inoculation of influenza virus disappeared (Sakagami et al., 1992c). When influenza virus was incubated with ^{125}I-labeled Fr. VI and subjected to sucrose density gradient centrifugation, the radioactivity derived from Fr. VI co-migrated with the band of influenza virus protein, suggesting an affinity between Fr. VI and influenza virus. These results indicate that lignin–carbohydrate complexes inactivate the influenza virus by directly binding to the virus (Sakagami et al., 1992c). The reason why lignin and "phenylpropanoid polymer" showed similar anti-HIV and anti-influenza virus activities may be that both of these substances have higher-order molecular structures that have high affinity for the viruses.

Lignin–carbohydrate complexes, isolated from the cones of various pine trees (*P. parviflora* Sieb. et Zucc., *P. densiflora* Sieb. et Zucc., *P. thunbergii* Parl., *P. elliottii* var. Elliottii, *P. taeda* L., *P. caribaea* var. Hondurenses, *P. sylvestris* L.) or from the seed shell of pine trees, such as *P. parviflora* Sieb. et Zucc. and *P. armadii* Franch, inhibited the proliferation of herpes simplex virus. On the other hand, glucans (Paramylon, Schizophyllan, TAK), *N*,*N*-dimethylaminoethylparamylon, sodium carboxymethylparamylon, sodium paramylon sulfate, carboxymethyl-TAK and PSK were inactive (Table 2). The anti-HSV activity of lignin–carbohydrate complexes became maximum when lignin was added at the time of virus adsorption to the cells (Fukuchi et al., 1989a). Tannin-related compounds also showed comparable anti-HSV activity (Fukuchi et al., 1989b). Due to the lack of a selective index (SI value), the anti-HSV activities of lignins and tannins have not been compared.

Lignin–carbohydrate complexes also inhibited the multiplication of rotavirus and enterovirus (Mukoyama et al., 1991). This antiviral activity was attributed to interference with virus adsorption, rather than to inhibition of virus replication after adsorption.

Table 2
Lignin–carbohydrate complexes (Frs. VI, VII) inhibited plaque formation by herpes simplex virus more potently than other antitumor polysaccharides

Treatment	% of inhibition
Fr. VI from	
Pine cone of *Pinus parviflora* Sieb. et Zucc.	100
Pine cone of *Pinus densiflora* Sieb. et Zucc.	100
Pine cone of *Pinus thunbergii* Parl.	100
Pine cone of *Pinus elliottii* var. Elliottii.	99
Pine cone of *Pinus taeda* L.	100
Pine cone of *Pinus caribaea* var. Hondurenses.	100
Pine cone of *Pinus sylvestris* L.	100
Seed shell of *Pinus parviflora* Sieb. et Zucc.	100
Seed shell of *Pinus armandii* Franch.	100
Fr. VII from	
Pine cone of *Pinus parviflora* Sieb. et Zucc.	89
Pine cone of *Pinus densiflora* Sieb. et Zucc.	97
Pine cone of *Pinus thunbergii* Parl.	100
Pine cone of *Pinus elliottii* var. Elliottii.	100
Pine cone of *Pinus taeda* L.	96
Pine cone of *Pinus caribaea* var. Hondurenses.	100
Pine cone of *Pinus sylvestris* L.	100
Seed shell of *Pinus parviflora* Sieb. et Zucc.	100
Glucans	
Paramylon	0
Schizophyllan	0
TAK	0
Chemically modified glucans	
N,*N*-Dimethylaminoethylparamylon	0
Sodium carboxymethylparamylon	0
Sodium paramylon sulfate	2
Carboxymethyl-TAK	0
Protein-bound polysaccharide	
PSK	0

Sample (10 μg/mL) was added to HSV-1-infected CV-1 cells.

2.6. Radical-scavenging activity

A close association between anticarcinogenic activity and antioxidant activity has been reported in a chemically induced mouse carcinoma system with low-molecular-weight polyphenols (Fujita et al., 1989; Tanaka et al., 1993; Tanaka, 1994; Makita et al., 1996). Fr. VI can scavenge several radicals such as O_2^-, hydroxyl radical (generated by the Fenton reaction) (Sakagami et al., 1992a, 1998; Satoh et al., 1999b), and NO [generated by NOC-7 (NO generator) in the presence of C-PTIO (a spin-trapping agent)] and hypochloric acid (demonstrated by the luminol chemiluminescence method) (Sakagami et al., 1995c). The O_2^--scavenging activity of Fr. VI (8–88 SOD unit/mg) (Sakagami et al., 1999b) was 1 order of magnitude lower than those of vitamin C (364 SOD unit/mg) (Sakagami et al., 2001) and tannins (2445–4706 SOD unit/mg) (Sakagami et al., 2001). The NO-scavenging activity of Fr. VI was lower than that of EGCG (Sakagami and Satoh, 2002).

2.7. Synergism between lignin and vitamin C

Vitamin C can exhibit either antioxidant or prooxidant activity, depending on the concentration (Sakagami and Satoh, 1997; Sakagami et al., 1997c). We found that i.v. administration of supraphysiological concentration of sodium 5,6-benzylidene-L-ascorbate (SBA) induced the degeneration of an inoperable lung patient and DAB-induced hepatocellular carcinoma (Sakagami et al., 1991). We explored a method for determining SBA by high-performance liquid chromatography (Sakagami et al., 1993a) and found that SBA was unstable and contained trace amounts of degradation products; i.e., sodium ascorbate and benzaldehyde (Sakagami et al., 1995b). Sodium ascorbate has been shown to induce apoptotic cell death (characterized by

internucleosomal DNA fragmentation, loss of cell-surface microvilli, chromatin condensation, production of apoptotic bodies and vacuolization) in human promyelocytic leukemia HL-60 cells, more potently than SBA and benzaldehyde (Kuribayashi et al., 1994; Tajima et al., 1998). This finding prompted us to similarly investigate vitamin C. We found that ascorbate derivatives that produced ascorbyl radical induced apoptosis, as characterized by DNA fragmentation and an increase in the intracellular Ca^{2+} concentration. On the other hand, ascorbate derivatives that did not produce radicals were inactive (Sakagami et al., 1996). This suggests that there is some connection between radicals production and cytotoxicity in ascorbates.

We next investigated the interaction between pine cone extracts of *P. parviflora* Sieb. et Zucc. and vitamin C. Various fractions of pine cone except for the most hydrophobic fraction (hexane extract) produced radicals under alkaline conditions (Fig. 1(a)), and hydrophobic fractions such as hexane and ether extracts did not enhance the radical intensity of ascorbate, whereas more hydrophilic fractions such as EtOAc, BuOH and H_2O extracts and the procyanidin fraction significantly enhanced the radical intensity of ascorbate (Fig. 1(b)). Lignin–carbohydrate complexes (alkali–lignin, lignin sulfonate, Fr. VI) were found to most strongly enhance the radical intensity of sodium ascorbate (Fig. 2(a)), which rapidly decayed in the presence of lignin–carbohydrate complexes (Fig. 2(b)). The rapid decay of ascorbyl radical in the presence of lignin may be due to the breakdown of ascorbic acid or to the consumption of ascorbyl radical. On the other hand, tannins, such as gallic acid, tannic acid and epigallocatechin gallate (EGCG), reduced the radical intensity of sodium ascorbate (Fig. 2(a)). Upon close examination, gallic acid and sodium ascorbate were found to counteract each other, reducing the radical intensity of their counterpart (Fig. 2(a)). Sodium ascorbate neutralized gallate radicals at a molar ratio of 1:100 (Satoh et al., 1999a).

The cytotoxic or apoptosis-inducing activity of lignin–carbohydrate complexes was much lower than those of tannins (Sakagami et al., 1995a, 2000b;

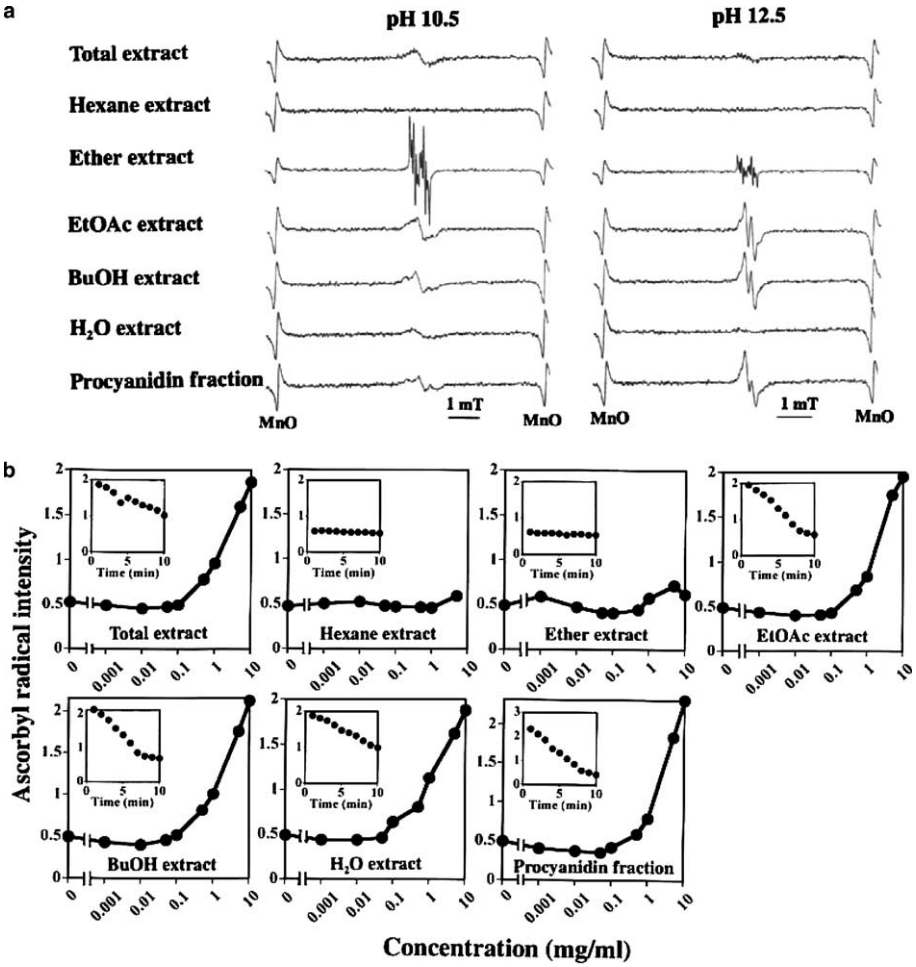

Fig. 1. Radical intensity (a) and ascorbyl radical modulating effect (b) of pine cone extract.

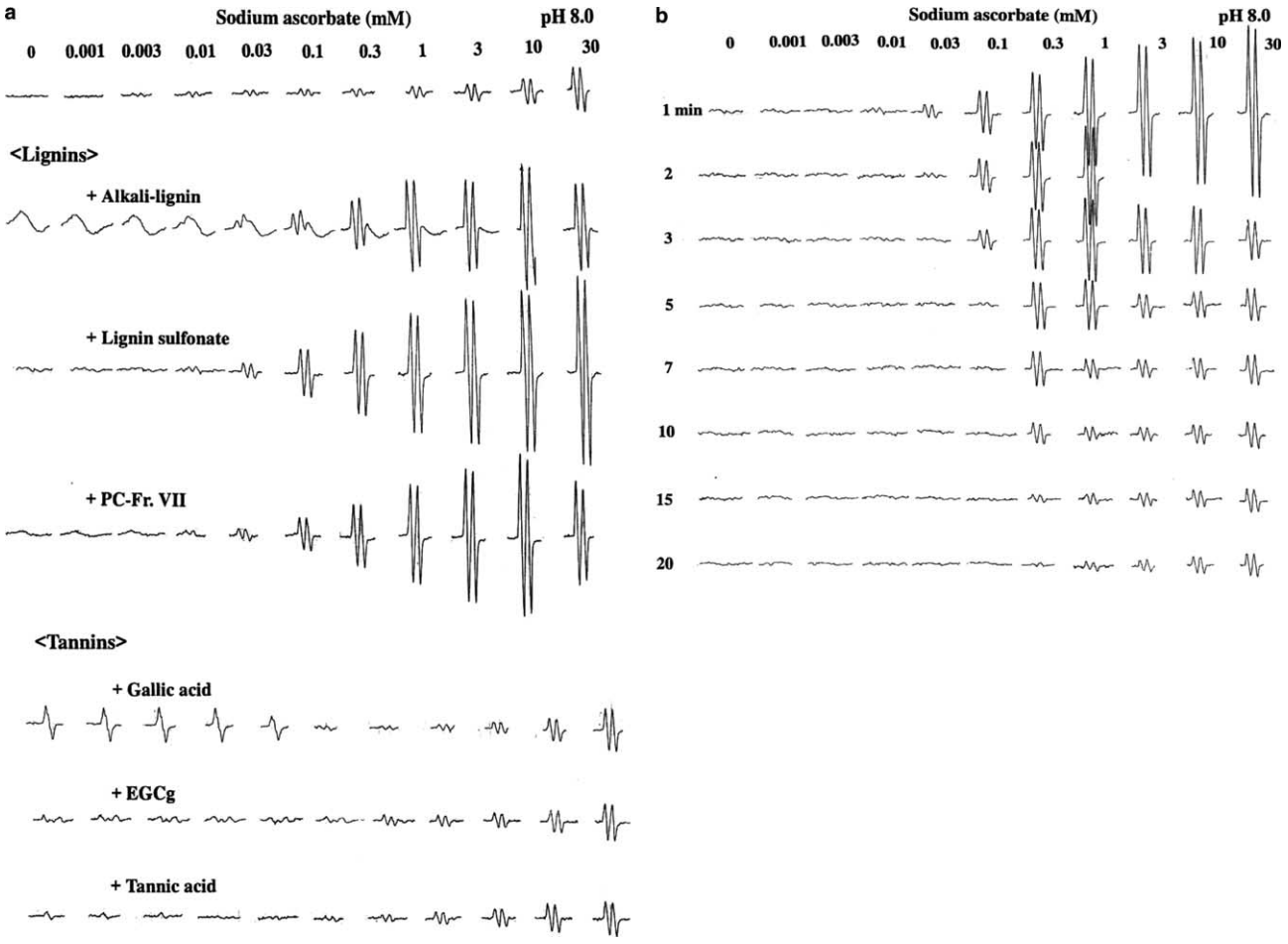

Fig. 2. Lignin–carbohydrate complexes enhanced ascorbyl radical, whereas tannin counteracted ascorbyl radical.

Paschka et al., 1998; Okabe et al., 1999; Ito et al., 2000), flavonoids with isoprenyl group(s) (C_5 units) (Fukai et al., 2000; Sakagami et al., 2000a; Hou et al., 2001; Shi et al., 2001; Shirataki et al., 2001), vitamin K_2 (menaquinone) geranylgeraniol (4 C_5 units) and geranylfarnesol (5 C_5 units) with isoprenyl groups (Ishihara et al., 2000). However, lignin–carbohydrate complexes showed higher cytotoxic activity against human oral tumor cell lines (human oral squamous cell carcinoma HSC-2, human salivary gland tumor HSG) than against human gingival fibroblast HGF (Jiang et al., 2001), suggesting that lignin–carbohydrate complexes have some tumor-specific cytotoxic action. Lignin–carbohydrate complexes, not only from pine cones, but also from Catuaba bark (Manabe et al., 1992), pine seed shell (Sakagami et al., 1992d), *A. nikoense* Maxim. (Sakagami et al., 1997a) and *C. Cuneata* Sieb. et Zucc. (Satoh et al., 1998), enhanced the radical intensity and cytotoxic activity of sodium ascorbate. On the other hand, tannins counteracted, rather than stimulated, the cytotoxic activity of sodium ascorbate (Satoh et al., 1999a) (Fig. 3).

We found that the addition of sodium ascorbate to the culture medium resulted in a rapid decrease in the oxygen concentration (monitored by polarography with a Clark-type electrode), possibly due to oxygen consumption during its pro-oxidation action, and that the simultaneous addition of lignin–carbohydrate complexes further enhanced the ascorbate-stimulated consumption of oxygen (Sakagami et al., 1997b). These data suggest that the synergistic enhancement of the cytotoxic activity of lignin–carbohydrate complexes and ascorbate might be due at least in part to the stimulated induction of hypoxia.

Lower concentrations of Fr. VI and sodium ascorbate showed radical-scavenging activity. The ability to scavenge O_2^- (generated by the hypoxanthine–xanthine oxidase reaction) was in the following order: sodium ascorbate > Fr. VI > ferulic acid. The combination of Fr. VI and sodium ascorbate further enhanced the O_2^--scavenging activity, whereas that of ferulic acid and ascorbate was not as effective. Fr. VI enhanced the DPPH-scavenging activity of sodium ascorbate more potently than ferulic acid (Fig. 4).

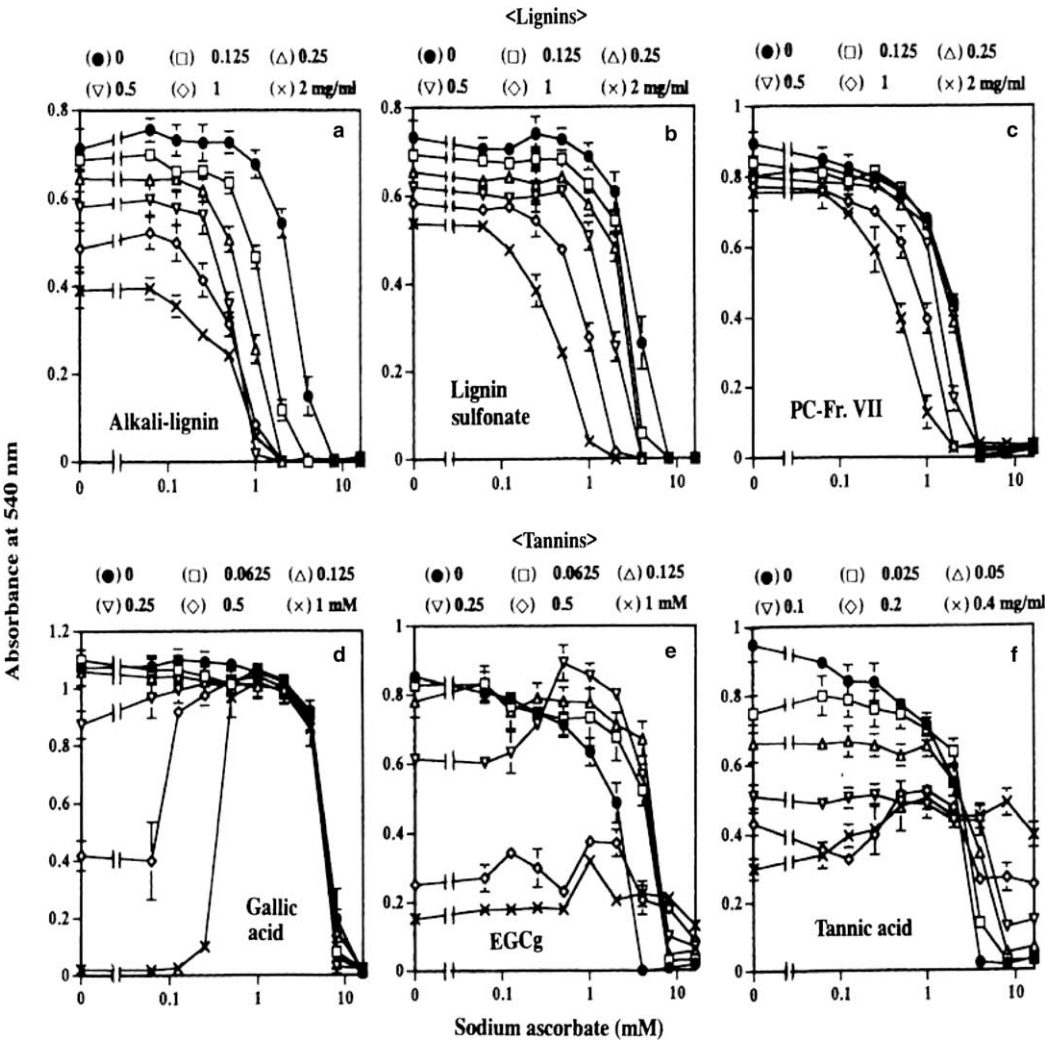

Fig. 3. Lignin–carbohydrate complexes, but not tannins, stimulated the cytotoxic activity of sodium ascorbate.

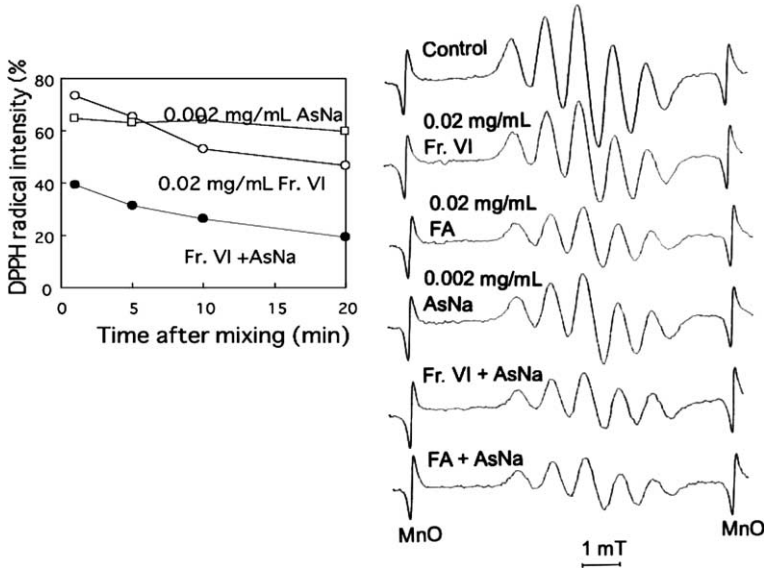

Fig. 4. Lignin–carbohydrate complex (Fr. VI) enhanced the DPPH-scavenging activity of vitamin C, more potently than ferulic acid.

Table 3
Among polyphenols, lignin–carbohydate complexes (Fr. VI, VII), but not phenylpropenoid monomers (FA, CA) or tannin-related compounds (EGCG, GA), stimulated the production of NO, ASN and CIT by Raw 264.7 cells, to an extent comparable to that attained with LPS

Treatment	Concentration (μg/mL)	NO (μM)	CIT (μM)	ASN (μM)
None (control)	0	0.0	44	0
Fr. VI	1	0.1	45	0
	10	8.4	53	23
	100	32.0	114	46
	1000	2.4	49	23
Fr. VII	1	0.0	40	0
	10	0.1	50	13
	100	19.4	80	24
	1000	13.1	96	0
FA	1	0.2	40	0
	10	0.2	44	0
	100	0.2	37	0
	1000	0.0	42	0
CA	1	0.3	42	0
	10	0.0	42	0
	100	1.4	43	0
	1000	3.4	0	0
EGCG	0.01 (mM)	0.0	37	0
	0.03	0.1	37	0
	0.1	0.2	35	0
	0.3	2.5	27	0
GA	0.01 (mM)	0.0	41	0
	0.03	0.0	41	0
	0.1	0.0	41	0
	0.3	1.4	35	0
LPS	0.1	32.7	136	27

2.8. Lignin–carbohydrate complexes, but not its precursors or other lower-molecular-weight polyphenols, stimulated NO and ASN production by activated macrophages

We used mouse macrophage-like Raw 264.7 cells, since this cell line has been popular for studies on macrophage activation and signal transduction. Lignin–carbohydrate complexes showed the lowest cytotoxicity against Raw 264.7 cells, followed by phenylpropanoid polymers and monomers (Suzuki et al., 2002a). Lipopolysaccharide (LPS), a well-known macrophage stimulator, activated Raw 264.7 cells to produce nitric oxide (NO) and citrulline (CIT) from arginine (ARG). Without LPS, about 13% of ARG is used to produce NO and CIT. LPS significantly reduced the consumption of most of amino acids except ARG, and about 87% of ARG is used to produce NO and CIT (Suzuki et al., 2002b). Similarly, lignin–carbohydrate complexes (Frs. VI, VII) stimulated the production of NO, CIT and ASN to levels comparable to those attained with LPS, whereas phenylpropenoids (such as ferulic acid and caffeic acid) and tannins (such as EGCG and gallic acid) were inactive (Suzuki et al., 2002a) (Table 3). Western blot analysis demonstrated that LPS stimulated the intracellular concentration of ASN synthetase (AS) (Suzuki et al., 2002b) (Fig. 5). This was due to stimulation of the expression of AS mRNA, and the stimulation of AS mRNA expression was increased with an increase in the glucose concentration in the culture medium. Fr. VII also increased the intracellular concentration of AS (Fig. 5). When the cells were incubated with Fr. VI, the electrophoretic mobility of AS was slightly delayed, possibly due to the binding of Fr. VI and AS (Fig. 5). Further experiments will be required to confirm this point. The biological significance of the increase in AS in Raw 264.7 cells is unclear. We found that AS expression declined during the TPA-induced differentiation of human promyelocytic leukemia HL-60 cells toward monocyte and macrophages (Hashimoto et al., unpublished data). Taken together, these findings suggest that AS activation may be involved in the activation of macrophages, but not in the differentiation toward macrophages.

These data demonstrate that lignin–carbohydrate complexes and tannin had different effects on macrophages. The former is stimulatory whereas the latter is inhibitory. Activation of macrophages is closely linked

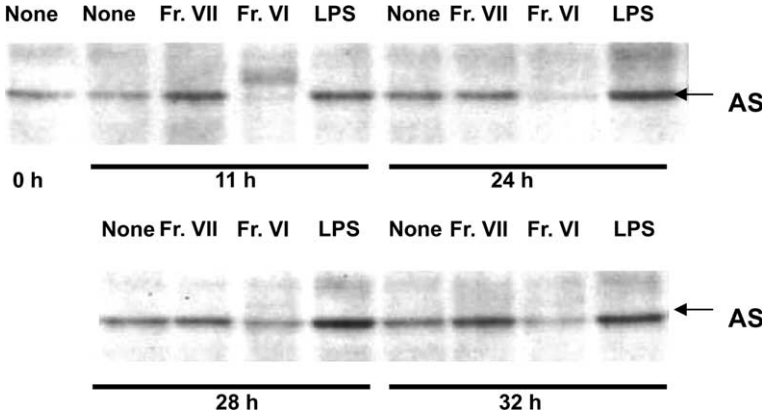

Fig. 5. Lignin–carbohydrate complexes (Fr. VI, VII) and LPS increased the intracellular concentration of asparagine synthetase.

to the expression of hypoxia-inducible factor (HIF)-1α, a transcription factor which turns on oxygen-providing systems by directing the expression of glycolytic enzymes, glucose transporter, erythropoietin and vascular endothelial growth factor (VEGF) (Pugh and Ratcliffe, 2003). This is supported by the observation that the knock-down of HIF-1 α significantly reduced the macrophage function such as phagocytosis and the production of inflammatory cytokines (Robert, 2003). EGCG has been reported to inhibit the production of VEGF, which plays a significant role in angiogenesis (Jung et al., 2001), and the infiltration of cancer cells (Garbisa et al., 2001). On the other hand, lignin–carbohydrate complexes stimulated the production of cytotoxic factor(s), and inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), and nitric oxide (NO) (Suzuki et al., 2002a,b) by mouse and human monocytes and macrophages (Sakagami et al., 1993b; Ikeda et al., 1988b). Further studies are needed to determine whether lignin–carbohydrate complexes and tannins positively or negatively modulate the expression of HIF-1 α in macrophages.

3. Conclusions

Moderately high-molecular-weight substances extracted from pine cones were identified as lignin with about 20% monosaccharide content. These lignin–carbohydrate complexes exhibited three unique biological activities. First, they showed immunopotentiating activity such as induction of antitumor, antimicrobial and antiparasite activities and the endogenous production of cytokines in mouse. Second, they showed a broad antiviral spectrum based on effects that depended on the polymeric phenylpropanoid character of the lignin and not the carbohydrate residues. Third, lignins or its carbohydrate complexes augmented the activities of vitamin C: radical-scavenging activity at lower concentration and apoptosis induction at higher concentration. Other polyphenols, such as tannins and flavonoids, showed much lower or even antagonistic activity. These data suggest that the lignin–carbohydrate complexes may be suitable for the prevention of viral-induced diseases, such as skin diseases, hepatoma and autoimmune diseases such as rheumatism. The synergistic effects of lignin–carbohydrate complex and vitamin C suggest that the former may be useful as a nutritional supplement. However, further investigation of the site of action of lignins is needed for future clinical application.

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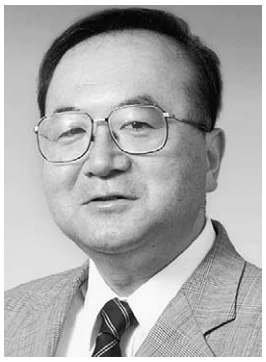

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