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Plant-Based Antioxidant Nanoparticles without Biological Toxicity

Kazuhiro Shikinaka,*[a] Masaya Nakamura,[b] Ronald R. Navarro,[b] and Yuichiro Otsuka[b]

Here, we present a function to derive non-deteriorated nanoparticulated lignin as an antioxidant without biological toxicity that is supplied through the simultaneous enzymatic saccharification and comminution of plants. The lignin exhibits an oxygen radical absorption capacity, even in its macromolecular nature. The non-deteriorated lignin nanoparticles never inhibit the biological activity of living things, despite their antioxidant nature. The oxygen radical absorption capacity of lignin is dependent on its botanical origin and monomeric structure. A stable organic radical in lignin is responsible for the antioxidant nature of non-deteriorated lignin. The organic radical of non-deteriorated lignin, which yields a distinct signal on electron spin resonance spectra, serves as a spin trap reagent that detects the emergence of short lifespan radicals as the change of radical concentration of the lignin. The presented discovery of non-deteriorated lignin will induce not only the industrial utilization of plant biomass polymers in pharmaceuticals and reagents, but also advance our scientific understanding of the antioxidant function of native lignin.

The utilization of natural resources, with the exception of petroleum, is a fundamental task for sustainable social development.[1] Plant biomass polymers, polysaccharides, and lignin are viewed as alternative resources of petroleum, owing to their production quantities in comparison to petroleum.[2] However, these resources have primarily been used as supplemental additives for the modification of petroleum materials. For example, lignin, a plant polyphenol, has been applied as a heatproof filler for petroleum-based materials.[3,4]

Some researchers anticipate the use of lignin as an antioxidant in the pharmaceutical industry, owing to the stable organic radicals of lignin in its π-conjugated phenolic framework.[5,6] However, the ordinary method to extract lignin from plants requires treatment with strong/toxic chemicals, such as acids, bases, and organic solvents, which prevents the application of lignin in the food/drink/medical industries, owing to its deterioration and contamination from harmful components during the extraction process. Furthermore, conventional extraction of lignin generally yields a low molecular weight. The low-molecular-weight nature of lignin enhances infiltration of the cells and mitochondria, which induces inhibition of biological activities (i.e. an antimicrobial effect).[6] Other synthetic low-molecular-weight antioxidants have also been difficult to use in medical studies, owing to their biological toxicity.[7] This has led to the development of antioxidant nanoparticles, which are macromolecules with antioxidant functional groups, such as nitroxyl radicals, as novel antioxidant without biological toxicity, because the infiltration of antioxidants to cells and mitochondria is inhibited.[8–12] These antioxidant nanoparticles have been also been recently tested for their therapeutic properties.[13,14]

We recently achieved the extraction of lignin through a physicobiological process that employs the simultaneous enzymatic saccharification and comminution (SESC) of plants.[15] In principle, SESC could allow the extraction of lignin under extremely mild conditions (e.g. 50 °C, pH 4.0–6.0, and 1 psi) without the need for strong and toxic chemicals. Therefore, the toxic byproducts (i.e. furan derivatives and low-molecular-weight phenols) and their reagents never emerge and contaminate the lignin during the SESC process,[15] with the lignin (hereafter denoted SESC lignin) obtained as a water-dispersed platelet nanoparticle.[16] Furthermore, the SESC lignin possesses a non-deteriorated nature, such that the extraction ratio of the aromatic components through nitrobenzene oxidation from SESC lignin is the same as in raw plants.[15]

SESC lignin is an amorphous nanoparticle, with a diameter of several tens of nanometers (Figure 1), which possesses a negative charge in pure water (e.g. the zeta potential of SESC lignin from Japanese cedar is −27.2 mV in pure water). The water-dispersed SESC lignin exhibits an oxygen radical absorption capacity, thereby demonstrating its antioxidant nature. The Trolox equivalents antioxidant capacity (TEAC) of SESC lignin extracted from Japanese cedar is 25.3 μmol TE g^{-1}. SESC lignin, therefore, has a definite absorption property of radical oxygen species (ROS) that is similar to low-molecular-weight antioxidants,[7] including its macromolecular (i.e. nanoparticulated) nature, owing to the presence of the chemical structure shown in Figure 1.

The TEAC value of SESC lignin depends on its botanical origin (e.g. the TEAC value of SESC lignin originating from rice straw is 22.7 μmol TE g^{-1}). Previous studies[21] have demon-

[a] Dr. K. Shikinaka
Research Institute for Chemical Process Technology
National Institute of Advanced Industrial Science and Technology
Nigatake, 4-2-1, Miyagino-ku, Sendai 983–8551 (Japan)
E-mail: kaz.shikinaka@aist.go.jp

[b] Dr. M. Nakamura, Dr. R. R. Navarro, Dr. Y. Otsuka
Forestry and Forest Products Research Institute
Matsunosato, 1, Tsukuba 305–8687 (Japan)

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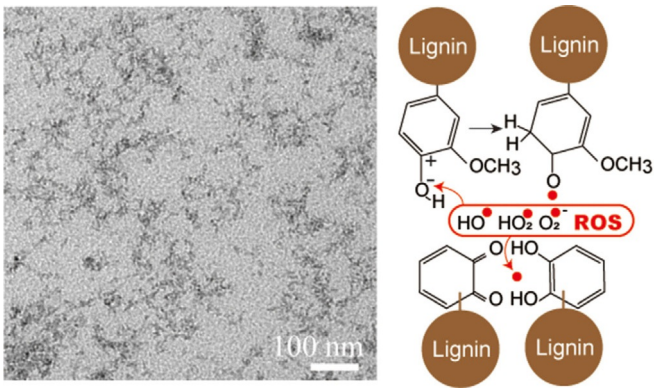

Figure 1. TEM image of SESC lignin (left) and candidates of the ROS-absorbed chemical structure in SESC lignin (right). The dark regions in the TEM image are the SESC lignin, as the SESC lignin is stained with gadolinium(III) acetate hydrate. The aryloxy- and semiquinone-type structure (lower right) that acts as a quinhydrone-type system (i.e. electron donor–acceptor complex) might emerge via oxidation and demethylation of the lignin by mechanical effects during SESC.[17–19].

strated that the monomer composition of lignin [i.e. the molar ratio of guaiacol (G), syringol (S), and phenol (C) units] is dependent on the plant species. Here, it is revealed that the lignin from cedar is essentially a homopolymer of vanillin (G/S/P = 1:0:0.05, with vanillin given a base value of 1). However, the lignin from straw is a copolymer of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde (G/S/P = 1:0.67:0.52).[21] The guaiacol and syringol units induce the antioxidant property of lignin in a phenolic framework.[19] The larger amount of syringol and phenol units in the phenolic framework of straw-derived lignin relative to that in cedar-derived lignin might cause the lower TEAC value of the straw-derived SESC lignin.

Previous studies have argued that the physical characteristics of extracted lignins (e.g. electrical properties[22]) only depend on their extraction method (e.g. kraft pulping, alkaline cooking, and glycerol thermal processing), with no dependence on their botanical origin. However, it seems that the extraction method of non-deteriorated lignin through SESC yields the original lignins of a plant species that exhibit antioxidant properties that are dependent on the plant species, such that further studies on SESC lignins will provide a better understanding of real lignin structure and functions, and reveal the true role and function of lignin in plants.

SESC lignin never inhibits biological activity despite its antioxidant nature. We previously revealed that the activity of (hemi)cellulase and sake yeast is maintained, even in the presence of SESC lignin, until the SESC and fermentation processes are complete.[15] Furthermore, SESC lignin never prevents the growth of bacteria and yeasts [i.e. the minimum inhibitory concentration (MIC) of SESC lignin is over 100 000 ppm]. These results indicate that the antioxidant property of SESC lignin does not affect living cells, only ROS.

The antioxidant property of SESC lignin is derived from its stable organic radicals,[19] which can be detected and estimated by electron spin resonance (ESR) experiments (Table 1 and Figure S1 a). Here, we emphasize that nanoparticulated SESC lignin shows clear ESR signals, even in a solid state at room temperature. The radical concentration of SESC lignin (10×10^{16} spin g^{-1}) is higher than that of ordinary organosolv lignin, which is a low-denatured lignin with a low-molecular-weight fraction (3×10^{16} spin g^{-1}).[23]

Table 1. Radical concentrations of powdered neat SESC lignin and solid mixture of SESC lignin and polymers estimated from the ESR spectra. The radicals from the polymers are undetectable, owing to the short lifespan of the ESR measurement. A small decrease in radical concentration of neat SESC lignin is reasonable for some depolymerization of lignin.[20]

	Neat sample [10^{16} spin g^{-1}]	Sample after thermal annealing [10^{16} spin g^{-1}]
Neat SESC lignin	10	7.1
SESC lignin + PEC	1.7	0.94
SESC lignin + CNF	2.1	5.4

SESC lignin also acts as a spin trap reagent, such as di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH), owing to its stable organic radicals, such that the SESC lignin can monitor the emergence of short lifespan radicals as the radical concentration of the SESC lignin changes. As shown in Table 1, the radical concentration of the SESC lignin and poly(ethylene carbonate) (PEC) solid mixture drastically decreases after annealing at 180 °C for 15 min. The guaiacol units in the SESC lignin trap the short lifespan oxygen radicals that originated from PEC thermal decomposition during thermal annealing (Figure 2).[24]

However, the radical concentration of the SESC lignin and cellulose nanofiber (CNF) solid mixture increases after annealing at 200 °C for 1 h. As shown in Figure 3, the hydroxyl radi-

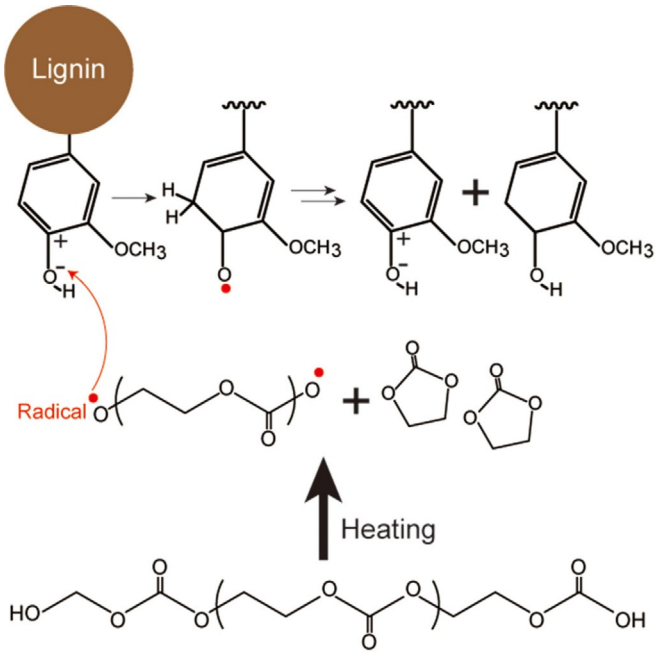

Figure 2. Schematic of radical trapping by the SESC lignin during PEC thermal decomposition. The amount of stable organic radicals is decreased according to this scheme, which yields a decreased radical concentration of the lignin–PEC mixture, by trapping radicals on the guaiacol units in SESC lignin.

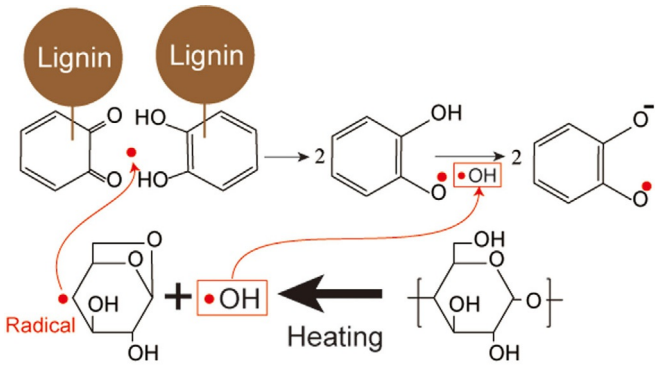

Figure 3. Schematic of radical trapping by the SESC lignin during cellulose thermal decomposition. The amount of stable organic radicals increases according to this scheme, which induces an increased radical concentration of the lignin–cellulose mixture by the generation of stable semiquinone radicals by trapping cellulose-derived radicals on aryloxy- and semiquinone-type structures that originated from partly oxidation of guaiacol units in lignin[19] and the reaction of hydroxyl radicals in lignin, as shown in Ref. [19].

cals and radicals formed during cellulose decomposition[25] are trapped in the aryloxy- and semiquinone-type structure of the lignin,[19] which causes the generation of additional stable semiquinone radicals. SESC lignin can, therefore, be utilized as a spin trap reagent that discerns the emergence of short lifespan oxygen and hydroxyl radicals.

In conclusion, we propose the utilization of SESC lignin as an antioxidant nanoparticle that acts as a non-toxic ROS scavenger and spin trap reagent, owing to the well-preserved phenolic functional groups of SESC lignin. Furthermore, the antioxidant property of SESC lignin is dependent on the botanical species from which the lignin is derived (i.e. the monomer components of lignin), which forms the first step in tailoring a function of lignin-based materials according to its chemical structure. The size of SESC lignin (several tens to hundreds of nanometers) is especially suitable for pharmaceuticals, because these antioxidant nanoparticles act on abnormal sites, such as cancer and inflammation sites, without accumulation on normal cells and mitochondria, such that the SESC lignin never exhibits antibiological properties despite its antioxidant nature. Furthermore, controls on the SESC period for plants yields size-tailored nanoparticulated lignin. SESC lignin can be prepared through an environmentally friendly process in principle (i.e. purification of plant components without hazardous petroleum-derived chemicals).[15] The antioxidant property of SESC lignin, which is based on its phenolic nature, therefore has a positive impact not only on the utilization of plants as new conceptual medical materials but also on the reduction of the environmental load caused by the utilization of limited petroleum-based resources. Furthermore, the antioxidant nanoparticles also reduce organ dysfunction and death in whole-body irradiated animals.[26,27] Therefore, the derivation of antioxidant nanoparticles from plants is anticipated to realize the novel medical concept of "Agricultural Pharmaceuticals". SESC lignin nanoparticles can also be used as spin trap reagents to detect short lifespan radicals that emerge through the thermal degradation of solid polymers. This fact also realizes the utilization of lignin as a novel functional macromolecule that is rare in petroleum-based products.

Experimental Section

Chemicals

SESC lignin was extracted from Japanese cedar and rice straw as a nanoparticle, with a diameter of approximately 40 nm, according to the method reported in our previous studies.[15,16] Ultrapure water was obtained by using a Milli-Q® Advantage A10® system (MilliporeTM, Eschborn, Germany) and employed throughout the study. 2,2′-Azobis (2-amidino-propane) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Aldrich. Fluorescein (FL; Na Salt) was obtained from SIGMA. Poly(ethylene carbonate) (PEC; $M_n = 39{,}000$) was purchased from Empower Materials, Inc. (New Castle, USA). The cellulose nanofibers were provided by the Forestry and Forest Products Research Institute.

Transmission Electron Microscopy (TEM)

TEM observations were performed using a JEM-2100 (JEOL, Tokyo, Japan) at an acceleration voltage of 200 kV. Portions of the lignin sample (5 μL) were dropped onto carbon-coated grids (Oken Shouji Co., Tokyo) whose surfaces had previously been rendered hydrophilic by glow discharge under reduced pressure. After 3 min, 2 wt/v% gadolinium(III) acetate hydrate was spread onto the grids, and the grids were then air dried. Digital TEM data were obtained using a slow-scan charge-coupled device camera (Gatan USC1000, Gatan, Inc.) and then converted into images with a frame size of 1024×1024 pixels. A cold finger and a cold trap cooled with liquid nitrogen were used to prevent sample contamination by the electron beams.

Zeta Potential Estimation

The zeta potential of the colloidal dispersion and solution of SESC lignin (0.068 wt/v%) was measured using Stabino® (Particle Metrix GmbH, Meerbusch, Germany) at 25 °C.

TEAC Estimation

The TEAC analyses were conducted by using a multi-grating microplate reader SH-9000Lab (CORONA Electric). Fluorescence filters, with an excitation wavelength of 485 nm and an emission wavelength of 520 nm, were used. The 96 well microplates (Coster No. 3792) used in the analyses were purchased from Corning. The pure-water dispersions of SESC lignin were centrifuged at 10000× *g* for 10 min. The supernatant was removed and then diluted as needed for the TEAC analyses. All reagents were prepared with a 75 mM phosphate buffer (pH 7.4) at 37 °C. FL (final concentration of 30.6 nM) was used as a target of the free radical attack and AAPH (final concentration of 31.25 mM) was used as a peroxyl radical generator in the final assay mixture (0.2 mL total volume). Trolox (6.25, 12.5, 25, and 50 μM) was used as the control standard. The microplate was incubated at 37 °C for 20 min before an aliquot of AAPH solutions was added to each well as peroxyl generators to start the reaction. The microplate reader was programmed to record the fluorescence of FL every minute after the addition of AAPH. All measurements were expressed relative to the initial reading. The final TEAC values were calculated by using a linear regres-

sion between the Trolox concentration (μM) and the net area under the FL decay curve. The linear regression was determined over the 6.25–50 μM Trolox concentration range. The data were analyzed by using Microsoft Excel. The TEAC values were expressed as micromolar Trolox equivalents (TE) per 100 mL (μM TE/100 mL).

MIC Measurement

The pure-water dispersed SESC lignin was purified by using a membrane filter with a 0.2 μm pore size. The purified samples were diluted in a Mueller Hinton Broth to a certain concentration. The fungus liquid (*Escherichia coli*; NBRC 3972) was cultured at 35 °C for 24 h on a soybean-casein digest agar (final number of fungi: 10^6 mL^{-1}). The 4.95 mL test samples (SESC lignin) and reference (pure water) were mixed with 0.05 mL fungal liquid, and the mixtures were then cultured at 35 °C for 24 h for the MIC value estimations. Fungal growth was observed in all the samples, even at 100 000 ppm SESC lignin.

ESR Spectral Measurements

The ESR spectra were recorded for the samples included in the ESR tubes at 9.4 GHz and 20 °C on the Bruker E500 (Bruker, Co., UK) in continuous-wave (CW) mode. The microwave power, scan width, and modulation amplitude were 0.025 mW, 200 G, and 2.0 G, respectively. The radical concentration was determined from the calibration curve that was prepared by using the ESR spectra of DPPH. All the ESR spectra exhibited a peak at $g = 2.004$. The measured samples were as follows: neat powdered SESC lignin, neat powdered SESC lignin annealed at 180 °C for 15 min, solid mixture of SESC lignin and PEC, solid mixture of SESC lignin and PEC annealed at 180 °C for 15 min, solid mixture of SESC lignin and CNF, and solid mixture of SESC lignin and CNF annealed at 200 °C for 1 h.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antioxidants · lignin · nanoparticles · plant biomass · radical scavenger

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