

EVALUATION OF THE ABILITY OF AQUEOUS LIGNIN-CARBOHYDRATE COMPLEX EXTRACTED FROM BOTANICAL WASTE TO SCAVENGE REACTIVE OXYGEN SPECIES BY USING AN ELECTRON SPIN RESONANCE METHOD

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ABSTRACT

Lignin-carbohydrate complexes (LCCs) were extracted from botanical waste, *Quercus acutissima*, rice bran, peanut husks, tofu refuse, and used green tea leaves. The contents of the lignin and phenolic hydroxyl groups in the LCCs taken from these samples were positively correlated. The capacity of scavenging reactive oxygen species was evaluated by employing an electron spin resonance method for its superoxide dismutase (SOD)-like activity. The lignin content and the SOD-like activity of LCC from *Quercus acutissima* were much greater than those of other LCCs. These findings suggest that the SOD-like activity of LCCs depends on the lignin content.

Keywords: Lignin-carbohydrate complexes, SOD-like activity, botanical waste, ESR.

INTRODUCTION

Lignin, a structural component of the cell walls of vascular plants, is a natural polymer abundantly found in the biosphere. It is taken up as a dietary fiber when vegetables and fruits are consumed. Although most of the lignin is excreted without being digested, it is thought that part of it has some effect on the human body. In fact, the antitumor and antimicrobial activities of some types of lignin have been reported (Sakagami et al. 1991, 1998).

In this research, the ability of lignin obtained from botanical waste to scavenge reactive oxygen species (ROS) was evaluated. ROS is a general term for highly reactive molecules derived

from oxygen, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. It is thought that the overproduction of ROS may play a role in various diseases, including inflammation, cancer, and epilepsy (Oyanagui et al. 1988; Kensler et al. 1983; Yokoyama et al. 1992). Therefore it is important to investigate the ability of lignin to scavenge ROS.

A superoxide radical is a typical ROS. Superoxide dismutase (SOD) is a specific enzyme that dismutates this superoxide radical. The dismutation rate by SOD occurs more rapidly than in the spontaneous process of becoming disproportionate (McCord and Fridovich 1969). Based on the superoxide radical concentration in a mixture of a sample and a solution that includes a superoxide

generating system, the superoxide scavenging activity of the sample can be estimated for a "SOD-like activity" (Kaneyuki et al. 1999). The electron spin resonance (ESR) method is a unique technique for the specific detection of electron spins of radicals. The hypoxanthine (HPX)/xanthine oxidase (XOD) system is commonly used for generating a superoxide radical. Because the HPX/XOD system is used in a water system, target samples should be water-soluble; therefore the SOD-like activity of a water-soluble component of lignin, the lignin-carbohydrate complex (LCC) from botanical waste, was evaluated by using an ESR spectrometer.

EXPERIMENTS

Preparation of lignin and lignin-carbohydrate complex

LCCs were extracted from botanical waste, the woody part of *Quercus acutissima*, rice bran, peanut husks, tofu refuse, and used green tea leaves with the stalk.

The samples were extracted in a powder form by using Soxhlet's extractor with 100% acetone (Wako Pure Chemicals Co. Japan) and with a 90% (volume/volume, v/v) aqueous acetone solution. Each extraction was continued for 48 h. The residue was dried and extracted also by using the Soxhlet's extractor with an 80% (v/v) aqueous dioxane (Wako Pure Chemicals Co. Japan) solution for 72 h. The residue resulting from lignin extraction by these procedures was again extracted with hot water (60°C) for 1 h. The extract was precipitated from five volumes of ethanol to yield the LCC.

The LCCs of *Quercus acutissima*, rice bran, peanut husks, tofu refuse, and green tea are referred to here as QLCC, RLCC, PLCC, TLCC, and GLCC, respectively.

Estimation of contents of lignin and phenolic hydroxyl group

The contents of lignin were determined by an acetyl bromide method (Johnson et al. 1961). The contents of the phenolic hydroxyl group were estimated by ionization difference spec-

trum (Goldschmid 1954). The percentage of phenolic hydroxyl groups in LCC was obtained from the formula: $(17\Delta\alpha_{\max})/4100 \cdot 100$, where $\Delta\alpha_{\max}$ is the differential absorbance for each $\text{g/l} \cdot \text{cm}$ at 280 nm. For these measurements, a spectrophotometer (U-2000, Hitachi, Japan) was used. The error level of each determination was less than 10^{-2} .

Estimation of SOD-like activity

As a superoxide generation system, solution of 2 mM of HPX (Sigma Chemical Co. USA) and 0.4 unit/ml of XOD (milk origin, Roche, Germany) was prepared in a phosphate buffer solution (PBS, 0.1 M, pH 7.4). The XOD solution was kept on crushed ice. SOD (human erythrocytes origin CuZn-SOD, 3900 units/mg, Sigma Chemical Co. USA) was dissolved in ultra-pure water in concentrations of 1.50, 2.25, 3.00, 3.75, and 4.50 units/ml. The samples were also dissolved in ultra-pure water. The concentrations of TLCC were 6, 10, 15, 20, and 30 mg/ml; for PLCC, 0.2, 1.0, 2.0, 5.0, and 10.0 mg/ml; for RLCC, 1.0, 2.0, 3.3, 5.0, and 10.0 mg/ml; for GLCC, 0.10, 0.25, 0.50, 0.75, and 1 mg/ml; and for QLCC, 0.01, 0.02, 0.03, 0.05, and 0.10 mg/ml. As a spin-trapping reagent, 5-dimethyl-1-pyrroline-1-oxide (DMPO, 9.2 M, Labotec. Co. Japan) was used.

An XOD (50 μl) solution was added to a mixed solution of DMPO (15 μl), HPX (50 μl), SOD or sample (35 μl), and PBS (50 μl). Immediately after the mixture was stirred, it was placed in a capillary tube at the center of the cavity resonator of an ESR spectrometer (JES-TE 200, JOEL, Japan). ESR measurements were started 60 s after XOD was added. The conditions for measurement were: microwave power, 8 mW; center magnetic field, 336.8 mT; magnetic field sweep width, 10 mT; magnetic field sweep time and time constant, 120 s and 0.1 s; and magnetic field modulation width, 0.1 mT at 100 kHz.

The ESR spectrum thus obtained was identified with a DMPO-superoxide adduct (DMPO-OOH) by measuring the hyperfine coupling

constant, which was $a^N = 1.42$ mT, $a^H_\beta = 1.14$ mT, and $a^H = 0.13$ mT (Fig. 1). The ESR signal intensity was derived from the ratio of the peak height of the low magnetic field component of an ESR spectrum (I_s in Fig. 1) to that of an external standard marker (I_M in Fig. 1). The ESR measurements were also made without the SOD or the sample solution. “I” and “ I_0 ” were defined as ESR signal intensities with and without the SOD or sample solution, respectively. The relationship between “ $I_0/I-1$ ” and the SOD or the sample concentration was plotted. SOD-like activity was obtained on the basis of the rate of concentration of SOD to that of the sample, which gave $I_0/I-1 = 1$ in the plotted curve.

RESULTS AND DISCUSSION

As shown in Fig. 2, the contents of lignin (weight/weight, w/w) for TLCC, PLCC, RLCC, GLCC, and QLCC were 9.8%, 4.4%, 9.4%, 10.7%, and 30.1%, respectively. As shown in Fig. 3, the contents of the phenolic hydroxyl

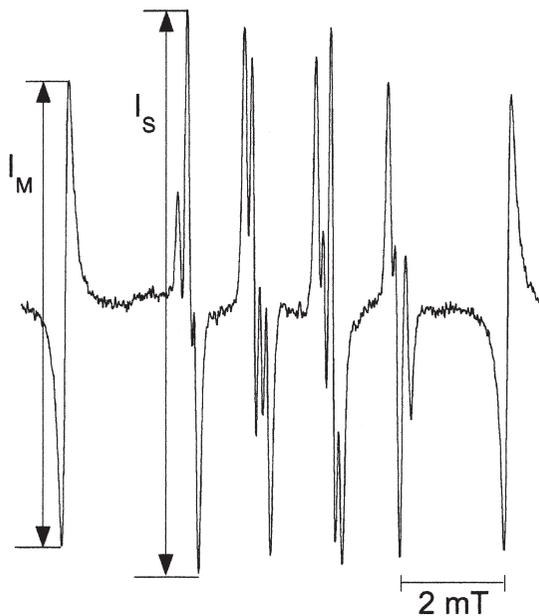


FIG. 1. An example of an ESR spectrum of DMPO-OOH. The ESR signal intensity was derived from the ratio of the peak heights of the low magnetic field component of an ESR spectrum (I_s) to an external standard marker (I_M).

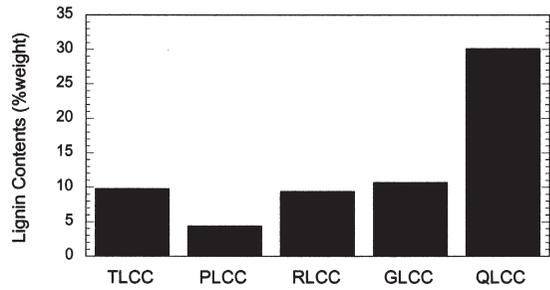


FIG. 2. Lignin contents of LCCs.

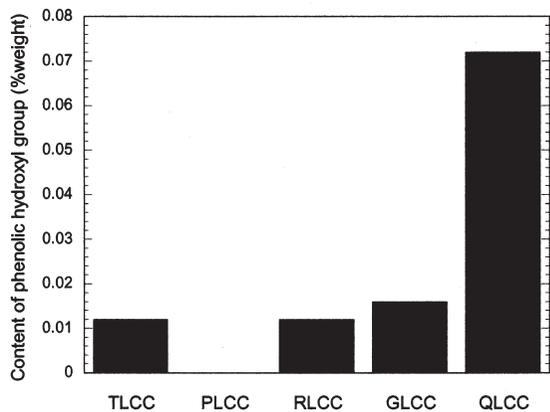


FIG. 3. Contents of phenolic hydroxyl group in LCCs.

groups for TLCC, RLCC, GLCC, and QLCC were 0.011%, 0.016%, 0.016%, and 0.077%, respectively. Nothing was detected for PLCC. The contents of phenolic hydroxyl groups and lignin were positively correlated (correlation coefficient = 0.999).

As shown in Fig. 4, the SOD-like activities of TLCC, PLCC, RLCC, GLCC, and QLCC were 0.20 ± 0.01 , 0.73 ± 0.03 , 4.04 ± 0.06 , 6.04 ± 0.50 , and 209.32 ± 4.88 units/mg, respectively (values are mean \pm standard error from 5 independent determinations). The value for QLCC is much greater than those of the other LCCs. Catechin, tannin, and flavonoid groups are well-known components with SOD-like activity in plants (Kaneyuki et al. 1999; Kim et al. 1995; Fukuda et al. 2003). Although LCC obtained by the present method includes monosaccharides (such as arabinose, xylose, and mannose) as impurities (Björkman 1956; Watanabe et al. 1987), the

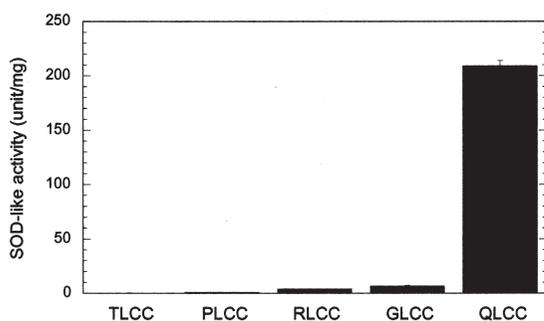


FIG. 4. SOD-like activity of LCCs.

components with the SOD-like activity described above were excluded during the LCC preparation because they had been dissolved well in ethanol. We think that the SOD-like activity shown in this study is due to the structure of LCC.

In the SOD-like activity of polyphenol, such as flavonoid, the activity increases with the number of hydroxyl groups in a benzene ring (Husain et al. 1987; Puppo 1992; Yoshiki et al. 1995). If it is assumed that the number of phenolic hydroxyl groups in a benzene of lignin in LCC affects the SOD-like activity, one may expect the SOD-like activity of the lignin to be low because the number is one. In fact, the SOD-like activity of LCC was very low (except for QLCC, Fig. 4).

These results show that the SOD-like activity and the content of lignin for QLCC were much greater than for the other LCCs (Figs. 2 and 4). As noted above, the contents of the phenol hydroxyl group for LCC increases as the lignin content increases. These findings suggest that the SOD-like activity of LCC was influenced by the amount of lignin (i.e., quantity of the phenol hydroxyl groups). The highly concentrated phenol hydroxyl group in LCC may produce a similar effect on multiple phenol hydroxyl groups in a benzene ring because of the complex three-dimensional structure of lignin in LCC.

The SOD-like activity of extracts from a mangrove plant (*Ceriops decandra* (Griff.) Ding Hou), maple (*Acer nikoense* Maxim), and thorn apple (*Crataegus Cuneata* Sieb et. Zucc.) has already been reported (Sakagami et al. 1998; Satoh et al. 1998a; 1998b). In those reports, the

SOD-like activities of ethanol, hot water, and alkaline extracts in the HPX/XOD system were investigated by using the ESR spectrometer. It is believed that, based on the extraction methods described in those reports, their extracts were a mixture of catechin, lignin, tannin, and LCC. Furthermore, it was not clear exactly how much lignin was in the extracts, and it was not certain which components in those extracts contributed to the SOD-like activity. In this study, the relationship between the lignin and the SOD-like activity could be clarified by excluding catechin and tannin from the samples and investigating the concentration of lignin in LCC.

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