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Free Radical Scavenging Activity of Chitooligosaccharides by Electron Spin Resonance Spectrometry

PYO-JAM PARK, JAE-YOUNG JE, AND SE-KWON KIM*

Department of Chemistry, Pukyong National University, Busan 608-737, Korea

The radical scavenging effects of chitooligosaccharides (COSs) on hydroxyl radical, superoxide radical, alkyl radical, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were investigated using a spin-trapping electron spin resonance (ESR) method and compared with the ESR signal intensity. COSs exhibited strong scavenging activity on hydroxyl radical and superoxide radical and weak scavenging activity on alkyl radical and DPPH radical. The radical scavenging activity of COSs increased with increment of concentration, and it was also dependent on molecular weight. These results suggest that the scavenging activity of COSs is dependent on their molecular weights and tested radicals.

KEYWORDS: Chitooligosaccharides; electron spin resonance; free radical; radical scavenging activity

INTRODUCTION

Chitosan is an *N*-deacetylated derivative of chitin, which is a naturally abundant mucopolysaccharide and distributed in the shell of crustaceans, in the cuticle of insects, and also in the cell wall of some fungi and microorganisms. It has received considerable attention for its commercial applications in biomedical, food, and chemical industries (*1*, *2*). However, chitosan shows its biological activity only in acidic medium because of its poor solubility above pH 6.5. Therefore, recent studies on chitosan have attracted interest for converting it to chitooligosaccharides (COSs), because the COSs not only are water-soluble but also possess versatile functional properties such as antitumor activity (*3*, *4*), immunostimulating effects (*5*, *6*), and antimicrobial activity (*7*−*12*).

Free radical scavengers are preventive antioxidants. Antioxidants can act at different levels in an oxidative sequence. This may be illustrated by considering one of the many mechanisms by which oxidative stress can cause damage by stimulating the free radical chain reaction of lipid peroxidation. Free radical chain reactions within a material could be inhibited by adding chemicals that retard the formation of free radicals, by introducing substances that compete for the existing radicals and remove them from the reaction medium. Lipid oxidation is of great concern to the food industry and consumers because it leads to the development of undesirable off-flavors and potentially toxic reaction products (*13*). Synthetic antioxidants such as butylated hydroxyanisole, butylatd hydroxytoluene, *tert*-butylhydroquinone, and propyl gallate may be added to food products to retard lipid oxidation (*14*). However, use of synthetic antioxidants in food products is under strict regulation due to the potential health hazards caused by such compounds (*15*). In addition, free radical-mediated modification of DNA, proteins, lipids, and small cellular molecules is associated with a number of pathological processes, including atherosclerosis, arthritis, diabetes, cataractogenesis, muscular dystrophy, pulmonary dysfunction, inflammatory disorders, ischemia−reperfusion tissue damage, and neurological disorders such as Alzheimer's disease (*16*). Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers.

In the present study, we investigated the radical scavenging activity of COSs as an antioxidant on hydroxyl, superoxide, alkyl, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals using electron spin resonance (ESR).

MATERIALS AND METHODS

Materials. Chitosan (degree of deacetylation, 93%; average molecular weight, 280 kDa; viscosity, 13 cP) prepared from crab shell chitin was donated by Kitto Life Co. (Seoul, Korea). Chitosanase (35000 units/g of protein) for the preparation of COSs was from *Bacillus* sp. (Amicogen Co., Jinju, Korea). The ultrafiltration membrane (UF) reactor (Minitan) system and membranes for the fractionations of COSs, based on molecular weight, were purchased from Millipore Co. (Bedford, MA). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN), hypoxanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade available commercially.

Preparation of COSs Using an UF Membrane Bioreactor. Chitosan solution (1%) was prepared by dispersing 100 g of chitosan in 1.0 L of distilled water while stirring with 550 mL of 1.0 M lactic acid and making the final volume up to 10 L with distilled water. The pH of the solution was adjusted to 5.5 using saturated $NaHCO_3$ solution. COSs were prepared by continuous hydrolysis of chitosan in an UF membrane reactor system connected to an immobilized enzyme column reactor in which chitosanase from *Bacillus* sp. (Amicogen Co.) was adsorbed according to our previous method (*17*). Four different UF membranes ranging in molecular weight cutoffs (MWCO) of 10, 5, 3, and 1 kDa were used for fractionation into five kinds of COSs. The five kinds of COSs prepared in the system were COS I, oligosaccharides that are not passed through the 10 kDa membrane; COS II, oligosac-

* Author to whom correspondence should be addressed (telephone +82-51-620-6375; fax +82-51-628-8147; e-mail sknkim@mail.pknu.ac.kr).

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charides that are passed through the 10 kDa membrane but not through the 5 kDa membrane; COS III, oligosaccharides that are passed through the 5 kDa membrane but not through the 3 kDa membrane, COS IV; oligosaccharides that are passed through the 3 kDa membrane but not through the 1 kDa membrane; and COS V, oligosaccharides that are passed through the 1 kDa membrane. COSs recovered were lyophilized on a freeze-dryer for 5 days.

Hydroxyl Radical Assay. Hydroxyl radicals were generated by Fenton reaction and reacted rapidly with nitrone spin trap DMPO: the resultant DMPO−OH adduct was detectable with an ESR spectrometer (*18*). The ESR spectrum was recorded 2.5 min after mixing in a phosphate-buffered solution (pH 7.4) with 0.2 mL of 0.3 M DMPO, 0.2 mL of 10 mM $FeSO_4$, and 0.2 mL of 10 mM H_2O_2 using a JES-FA ESR spectrometer (JEOL, Tokyo, Japan) set at the following conditions: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5; temperature, 298 K.

Superoxide Radical Assay. Superoxide radicals were generated from a hypoxanthine−xanthine oxidase system (*18*). Hypoxanthine (50 μL, 4 mM) was mixed with 30 μL of PBS, 50 μL samples, 20 μL of DMPO (4.5 M), and 50 μL of xanthine oxidase (0.4 unit/mL). The reaction mixture was transferred into a 100 μL Teflon capillary tube. After 45 s, the ESR spectrum was recorded using an ESR spectrometer. Manganese oxide was used as an internal standard. Experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 4 mW; gain, 6.3×10^5; temperature, 298 K.

Alkyl Radical Assay. Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures containing 10 mmol/L AAPH, 10 mmol/L 4-POBN, and indicated concentrations of tested samples were incubated at 37 °C in a water bath for 30 min (*19*) and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on a JES-FA ESR spectrometer. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5; temperature, 298 K.

DPPH Radical Assay. DPPH radical scavenging activity was measured using the method described by Nanjo et al. (*20*). An ethanol solution of 60 μL of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μmol/L) in ethanol solution. After mixing vigorously for 10 s, the solutions were then transferred into a 100 μL Teflon capillary tube and fitted into the cavity of the ESR spectrometer. The spin adduct was measured on an ESR spectrometer exactly 2 min later. Measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5; temperature, 298 K.

RESULTS AND DISCUSSION

The chitooligosaccharides were successfully prepared using an UF membrane bioreactor system with four different membranes, and the molecular weight profiles of each oligosaccharide showed a distinct decrease of molecular weights according to the pore size of membranes used (date not shown).

Hydroxyl radicals generated in the Fe^{2+}/H_2O_2 system were trapped by DMPO, forming a spin adduct detected by ESR spectrometer, and the typical 1:2:2:1 ESR signal of the DMPO−OH adduct was observed as shown in **Figure 1**. In addition, background signals are present in **Figure 1**. They may be due to the paramagnetic impurities contained in unpurified commercial DMPO (*21*). The height of the third peak of the spectrum represents the relative amount of DMPO−OH adduct. After the addition of COS IV, the decrease of the amount of DMPO−OH adduct was shown on the ESR spectrum. COS IV was the most effective scavenging activity among tested agents, and it scavenged ∼95% of the hydroxyl radical signals at 0.075 mg/mL. In addition, the scavenging activity increased with the concentration of the most COSs (**Table 1**). The superoxide scavenging activity of COSs was determined using the hypoxanthine−xanthine oxidase system as a superoxide source. COSs suppressed the signals of the superoxide anion−DMPO adduct on ESR charts. In addition, an unknown species is present together with the DMPO−OOH radical and the Mn^{2+} signals. Finkelstein et al. (*22*) reported that the unknown species is the DMPO−OH radical, which is known to be produced by decomposition of the DMPO−OOH adduct. The scavenging activity was increased with concentration increment of COS IV (**Figure 2**). As shown in **Table 2**, it was observed that the superoxide radical scavenging activities of COS I, COS II, COS III, COS IV, and COS V were 51.25, 56.82, 52.32, 66.85, and 45.74% at 1.0 mg/mL, respectively.

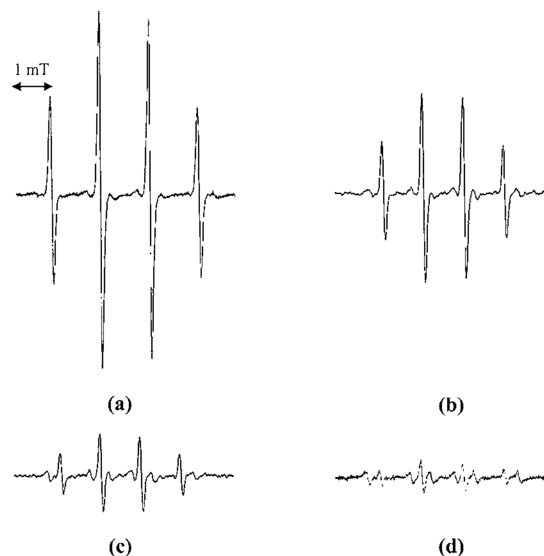

Figure 1. ESR spectra obtained in Fenton reaction system at various concentrations of COS IV: a, control; b, 0.025 mg/mL; c, 0.05 mg/mL; d, 0.75 mg/mL.

Table 1. Scavenging Activity of Chitooligosaccharides on Hydroxyl Radical

concn (mg/mL)	relative ESR signal intensity[a]				
	COS I[b]	COS II[c]	COS III[d]	COS I[e]	COS V[f]
0.025	65.28 ± 2.13	57.68 ± 2.05	55.63 ± 1.38	53.49 ± 0.83	55.24 ± 0.69
0.050	39.33 ± 0.56	27.21 ± 1.25	25.41 ± 0.72	18.95 ± 0.31	24.69 ± 0.49
0.075	20.51 ± 0.38	13.59 ± 0.47	15.16 ± 0.16	5.25 ± 0.09	13.97 ± 0.22

[a] Values represent the mean ± SD, $n = 3$. [b] COS I, the oligosaccharides that are not passed out through molecular weight cutoff (MWCO) 10 kDa membrane. [c] COS II, the oligosaccharides passed out through MWCO 10 kDa membrane but not passed out through 5 kDa membrane. [d] COS III, the oligosaccharides passed out through MWCO 5 kDa membrane but not passed out through 3 kDa membrane. [e] COS IV, the oligosaccharides passed out through MWCO 3 kDa membrane but not passed out through 1 kDa membrane. [f] COS V, the oligosaccharides passed out through 1 kDa membrane.

The alkyl radical spin adduct of 4-POBN/free radicals generated from AAPH at 37 °C for 30 min and the decrease of ESR signals were observed with the dose increment of COS IV (**Figure 3**). All tested COSs showed the alkyl radical scavenging activity, and the scavenging activity of COS IV increased from 44.76 to 79.11% with increasing concentrations from 1.25 to 5.00 mg/mL of alkyl radical (**Table 3**). The DPPH radical scavenging activity of COS IV is shown in **Figure 4**, and the radical scavenging activity was concentration-dependent. It was observed that 5 mg/mL of COS I, COS II, COS III, COS IV, and COS V scavenged 74.02, 92.11, 96.79, 96.03, and 95.31% of DPPH radical, respectively (**Table 4**). Generally, DPPH signals decrease when the odd electron of DPPH radical

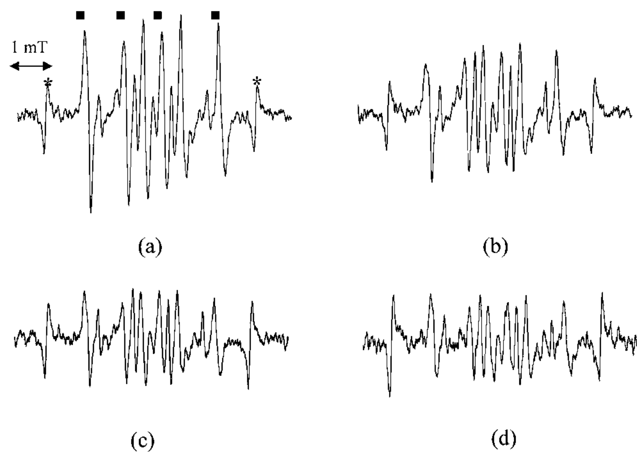

Figure 2. ESR spectrum obtained in hypoxanthine–xanthine oxidase system at various concentrations of COS IV: a, control; b, 0.25 mg/mL; c, 0.5 mg/mL; d, 1.0 mg/mL. Squares (■) represent position of the DMPO–OOH signal, and asterisks (∗) represent position of the Mn^{2+} signal.

Table 2. Scavenging Activity of Chitooligosaccharides on Superoxide Radical

concn (mg/mL)	relative ESR signal intensity				
	COS I	COS II	COS III	COS IV	COS V
0.25	93.38 ± 2.37	81.38 ± 1.99	72.59 ± 2.26	63.58 ± 1.84	70.86 ± 2.63
0.50	60.23 ± 1.59	63.17 ± 0.75	50.00 ± 1.64	38.04 ± 1.38	57.28 ± 1.22
1.00	48.75 ± 1.88	45.18 ± 1.43	47.68 ± 1.63	33.15 ± 1.26	55.26 ± 1.47

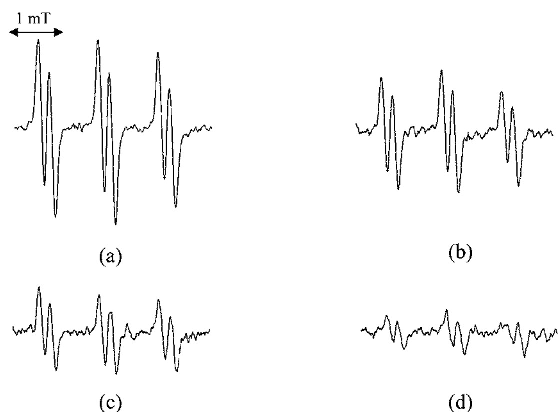

Figure 3. ESR spectrum observed during incubation of AAPH with 4-POBN at various concentrations of COS IV: a, control; b, 1.25 mg/mL; c, 2.50 mg/mL; d, 5.00 mg/mL. The incubation was done in a water bath containing 0.05 mol/L PBS, 10 mmol/L AAPH, and 0.1 mmol/L 4-POBN.

Table 3. Scavenging Activity of Chitooligosaccharides on Alkyl Radical

concn (mg/mL)	relative ESR signal intensity				
	COS I	COS II	COS III	COS IV	COS V
1.25	59.51 ± 1.13	54.82 ± 1.42	56.15 ± 1.29	55.24 ± 1.27	56.78 ± 1.33
2.50	42.55 ± 0.35	40.27 ± 0.88	38.32 ± 0.37	38.43 ± 0.63	39.57 ± 0.37
5.00	30.27 ± 0.42	27.99 ± 0.58	24.79 ± 0.42	20.89 ± 0.26	23.14 ± 0.18

is paired. The results indicate that all COSs examined were found to possess DPPH radical scavenging activity by pairing the odd electron of DPPH radicals. COSs have obvious scavenging activities on hydroxyl, superoxide DPPH, and alkyl radicals. In addition, the difference between tested radicals (DPPH radical, hydroxyl radical, and alkyl radical) is only a chemical difference. There is no enzymatic process involved

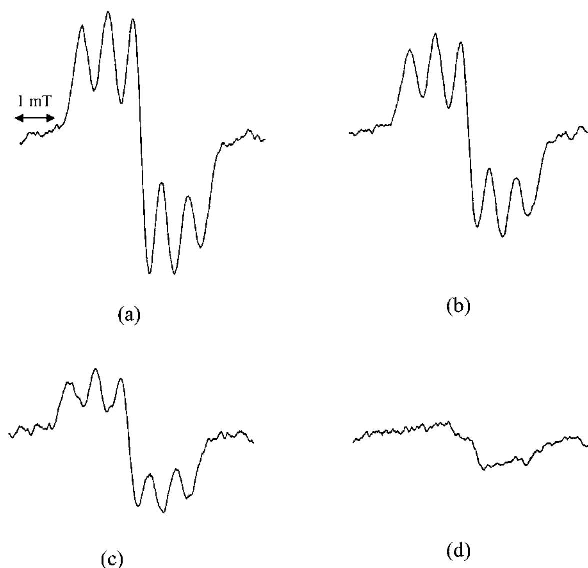

Figure 4. ESR spectrum obtained in an ethanol solution of 30 μmol/L DPPH at various concentrations of COS III: a, control; b, 1.25 mg/mL; c, 2.50 mg/mL; d, 5.00 mg/mL.

Table 4. Scavenging Activity of Chitooligosaccharides on DPPH Radical

concn (mg/mL)	relative ESR signal intensity				
	COS I	COS II	COS III	COS IV	COS V
1.25	85.49 ± 3.27	78.25 ± 2.48	70.00 ± 2.18	71.23 ± 2.59	72.64 ± 2.16
2.50	59.69 ± 2.22	49.55 ± 1.54	45.12 ± 1.38	46.26 ± 1.66	47.25 ± 1.57
5.00	25.98 ± 0.88	7.89 ± 0.04	3.21 ± 0.09	3.97 ± 0.09	4.69 ± 0.15

in the generation of these radicals. COSs exhibited the most effective activity on DMPO−OH scavenging.

Calliste et al. (*23*) extracted compounds with free radical scavenging activity from seven plants, and free radical scavenging activities were examined in different systems using ESR spectroscopy. Xie et al. (*24*) reported that water-soluble chitosan derivatives were prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium, and their scavenging activities were observed against hydroxyl radical. Matsugo et al. (*25*) reported that three different water-soluble chitosan derivatives by the acylation of chitosan inhibited thiobarbituric acid reactive substrate formation in *tert*-butylhydroperoxide and benzoyl peroxide induced lipid peroxidations. In the case of polysaccharide, Zheng et al. (*26*) reported that polysaccharide extracts from the plant *Saussurea involucrate* have free radical scavenging effects. In the present study, the scavenging activity of COSs increased with increment of concentration and was also dependent on molecular weight. These results suggest that the scavenging activity of COSs was dependent on their molecular weights and tested radicals.

In summary, the free radical scavenging activity of COSs was investigated on hydroxyl radical, superoxide radical, alkyl radical, and DPPH radical using ESR spectroscopy. COS IV (M_w 3000−1000 Da) showed the highest radical scavenging effect among the tested radicals, and the scavenging activity was dependent on their molecular weights and the tested radicals.

ABBREVIATIONS USED

AAPH, 2,2-azobis(2-amidinopropane) hydrochloride; COSs, chitooligosaccharides; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESR, electron spin

resonance; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; ROS, reactive oxygen species; UF, ultrafiltration membrane.

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