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Antioxidant activity of novel chitin derivative

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Abstract—Novel water-soluble chitin derivative was prepared by chemical modification to evaluate antioxidant activities by free radical scavenging potential using electron spin resonance spin trapping technique. Aminoethyl-chitin (AEC) exhibited free radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide, and peroxyl radicals. AEC quenched DPPH and peroxyl radical over 55% and 59% at 4 mg/mL, and also suppressed superoixde radical over 58% at 2 mg/mL. Especially, AEC was more active against hydroxyl radical, and scavenging ratio was 92.2% at 0.12 mg/mL. These results suggested that free amino group in the $-CH_2CH_2NH_2$ plays an important role in the free radical scavenging activity. In addition, cytotoxic effect of AEC was assessed using human lung fibroblast (MRC-5) cell line, and AEC showed less toxic against MRC-5.

Chitin 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. Chitin consists of 2-acetamido-2-deoxy-(1–4)-β-D-glucopyranose residues (*N*-acetyl-D-glucosamine units) that has intra- and intermolecular hydrogen bonds and is a water-insoluble material. It has drawn much attention on its potential application for medical devices or drug delivery such as a wound healing accelerator.[1] However, the poor solubility of chitin is probably the major limiting factor for its utilization. Therefore, special attention was paid to its chemically modified chitin derivatives with higher solubility in water such as carboxymethyl-chitin,[2] sulfated chitin,[3] sulfated carboxymethyl-chitin,[4] and glycosylated chitin.[5–7] In addition, their biological activities have been investigated for antiviral,[8] hemagglutination inhibition activity,[3] antimetastatic activity,[9] and antimicrobial activity.[7,10]

Major factor affecting biological aspects of chitin is free amino group at the C-2 position. Therefore, in the present study, aminoethyl-chitin with water-soluble property was prepared by grafting amino functionality onto chitin at the C-6 position, and antioxidant activity was investigated using electron spin resonance (ESR) spectroscopy.

Chitin (MW ~310 kDa, degree of deacetylation 10%) prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Chitin derivative was prepared according to our previous method.[11] In the FT-IR spectrum of the substituted aminoethyl group, a new peak was observed at 2950 cm^{-1} due to C–H stretching supporting the occurrence of the substitution. The ^1H NMR spectra of AEC in D_2O, a new chemical shift that appeared in the spectrum of aminoethyl-chitin derivative at 2.8 ppm, were assigned to protons of $-CH_2N$. The peak at 2.05 ppm was residual acetyl peak, and protons of pyranose unit superimpose the $-NH_2$ and $-OCH_2$ of aminoethyl group (2.9–3.6 ppm).[11] Degree of substitution of aminoethyl-chitin was 1.01 by elemental analysis and ^1H NMR data. The product was freeze-dried to give the aminoethyl-chitin (AEC: 0.403 g).

DPPH radical scavenging activity was measured using the method described by Nanjo et al.[12] An ethanol solution of 60 μL of each sample (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 μL quartz capillary tube, and the scavenging activity of AEC on DPPH radical was measured using a JES-FA ESR spectrometer (JEOL Ltd, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1× 1000; sweep time, 30 s.

Hydroxyl radicals were generated by iron-catalyzed Haber–Weiss reaction (Fenton-driven Haber–Weiss

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reaction) and the generated hydroxyl radicals rapidly reacted with nitrone spin trap 5,5-dimethyl-pyrroline *N*-oxide (DMPO).[13] The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Various concentrations of sample were mixed with DMPO (0.3 M), $FeSO_4$ (10 mM), and H_2O_2 (10 mM) in a phosphate-buffered solution (pH 7.2) and then transferred into a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1× 200; sweep time, 4 min.

Superoxide radical was generated by UV irradiation of a riboflavin/EDTA solution.[14] The reaction mixture containing 0.8 mM riboflavin, 1.6 mM EDTA, 0.8 M DMPO, and various concentrations of samples was irradiated for 1 min under UV lamp at 365 nm. The mixtures were transferred to the cavity of ESR spectrometer for measurement. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1× 1000; sweep time, 1 min.

Peroxyl radicals were generated by 2,2-azobis-(2-amidinopropane)-hydrochloride (AAPH). A phosphate-buffered saline (PBS) reaction mixture containing 10 mM AAPH, 10 mM α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), and sample with various concentrations was incubated for 30 min at 37 °C in a water bath[15] and then transferred to 100 μL quartz capillary tube. The spin adduct was recorded using an ESR spectrometer. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1× 1000; sweep time, 1 min.

The hydrogen peroxide scavenging assay was carried out according to the method of Muller.[16] Derivative with various concentrations (100 μL) and 10 mM hydrogen peroxide (20 μL) were mixed with 0.1 M phosphate buffer (pH 6.0, 70 μL) in a 96-microwell plate and incubated at 37 °C for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM 2,2-azinobis(3-ethylbenzthiazolin)-6-sulfonic acid (ATS) and 30 μL of peroxidase (1 U/mL) were mixed and incubated at 37 °C for 10 min and the absorbance was recorded with an ELISA reader at 405 nm.

The cytotoxic effect of AEC was assessed on a permanent fibroblast cell line derived from human lung (MRC-5) (ATCC CCL-171). MRC-5 fibroblasts were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C under a humidified atmosphere with 5% CO_2. Cells were seeded into 96-well plates at the density of 20,000 cells/well in DMEM supplemented with 10% FBS. After 24 h, various concentrations of AEC were treated, and controls were carried out with cells treated with an equivalent volume of serum-free medium without AEC. Cells were incubated for a further 24 h, and cytotoxic effect was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[17]

Antioxidant activity of AEC was tested for various free radical sources by ESR spin trapping technique. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity of an antioxidant. Figure 1 shows ESR spectra of DPPH when treated with AEC at various concentrations. An addition of AEC, the ESR signal was decreased with increase of AEC concentration. AEC scavenged DPPH radical over 55% at 4 mg/mL, and scavenging pattern was dose-dependent.

Hydroxyl radicals generated in Fe^{2+}/H_2O_2 system were trapped by DMPO forming spin adduct which could be detected by an ESR spectrometer, and the typical 1:2:2:1 ESR signal of the DMPO-OH adduct was observed. The hydroxyl radical scavenging activity of AEC was calculated by the height of the third peak of the spectrum which represents the relative amount of DMPO-OH adduct. After the addition of AEC, the decrease of the amount of DMPO-OH adduct was shown on the ESR spectra (Fig. 2). The ESR results showed that AEC suppressed about 92% of the hydroxyl radical at 0.12 mg/mL, scavenging pattern was the same as DPPH radical.

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals. Superoxide radical was generated by UV irradiation of a riboflavin/EDTA system[13] and trapped as DMPO spin adduct. AEC also quenched superoxide radical and scavenging ratio was 58.75% at 2 mg/mL (Fig. 3). AAPH can decompose to form carbon-centered radicals that can react swiftly with O_2 to yield peroxyl

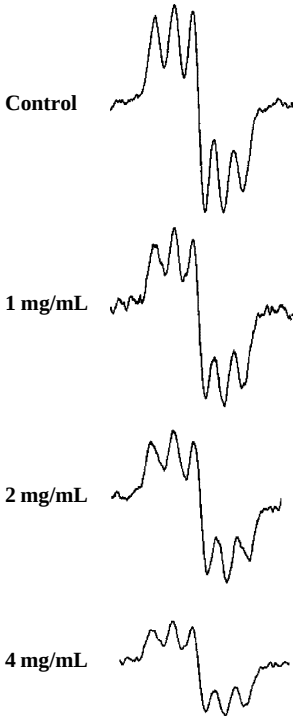

Figure 1. ESR spectra of DPPH in the absence and presence of AEC.

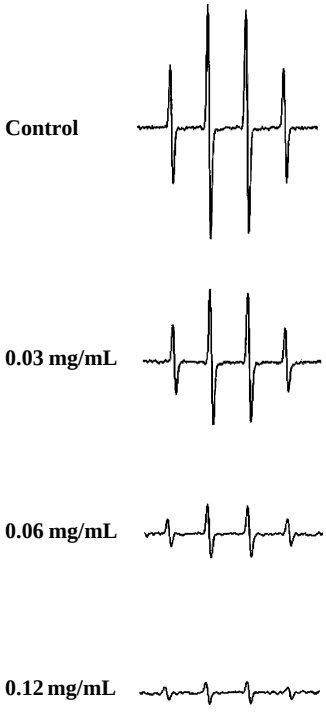

Figure 2. ESR spectra of hydroxyl radical in the absence and presence of AEC. Hydroxyl radicals were generated by iron-catalyzed Haber–Weiss reaction.

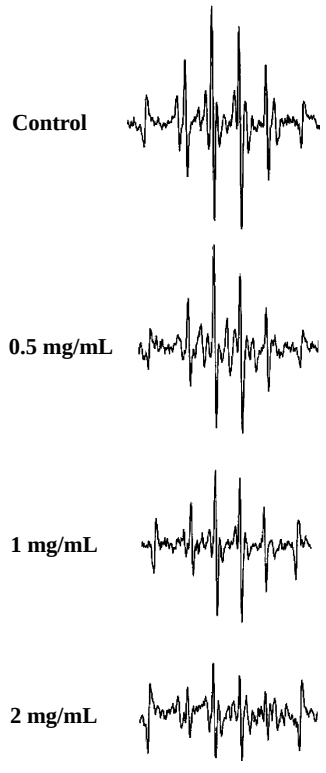

Figure 3. ESR spectra of superoxide radical in the absence and presence of AEC. Superoxide radical was generated by UV irradiation of a riboflavin/EDTA system.

radicals to stimulate lipid peroxidation.[18] The ESR results revealed that AEC suppressed peroxyl radical about 59.78% at 4 mg/mL (Fig. 4).

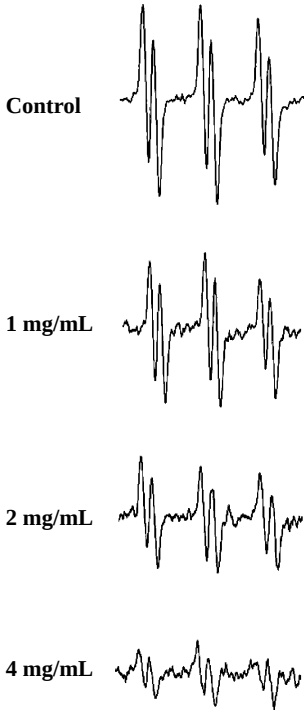

Figure 4. ESR spectra of peroxyl radical in the absence and presence of AEC. Peroxyl radicals were generated by AAPH.

Hydrogen peroxide, a reactive nonradical, is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals. Hydrogen peroxide scavenging activities of AEC are shown in Figure 5. AEC significantly scavenged over 68.6% on H_2O_2 at the concentration of 1 mg/mL, and scavenging pattern was carried out in a dose-dependent manner.

The cytotoxic effect of AEC on normal human lung fibroblast cell line was investigated. Cells were seeded into 96-well plates incubated for one day, and then various concentrations of AEC (125–1000 μg/mL) were treated. After MTT assay, the result showed that at the concentration of 1000 μg/mL exhibited over cell viability values of 90% (Table 1).

Park et al.[19] reported that free radical scavenging activities of hetero-chitosan with different degrees of deacetylation were dependent on the degree of deacetylation. The high degree of deacetylation chitosan, which exposed high amount of free amino group, showed the highest free radical scavenging activities. However, hetero-chitosan is water-insoluble, so it is very difficult for industrial and medical application in spite of their strong antioxidant activity. In the present study, we prepared AEC with water-soluble property by chemical modification and it also possessed strong antioxidant activity. The scavenging mechanism of the AEC on free radicals may be related to the fact that free radicals can react with the residual free amino groups NH_2 to form stable macromolecule radicals, and the NH_2 groups can form ammonium groups NH_3^+ by absorbing hydrogen ion from the solution.[20] This result suggested that

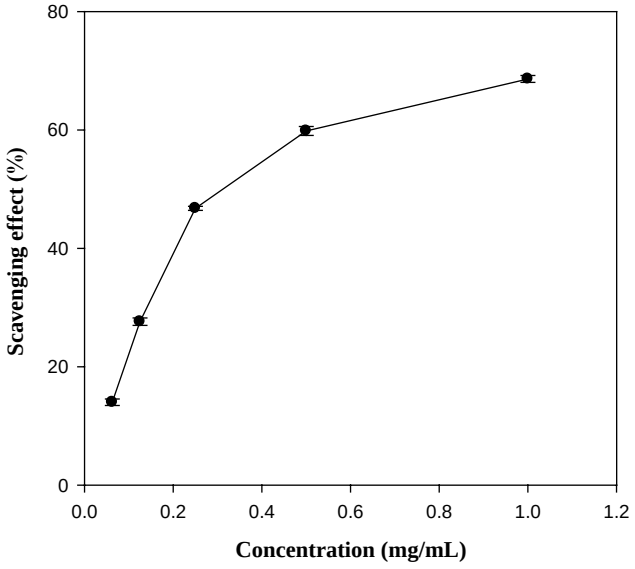

Figure 5. Hydrogen peroxide scavenging effect of AEC. Values represent means ± SE ($n = 3$).

Table 1. Cytotoxic effect of AEC on MRC-5

AEC (μg/mL)	Cell viability (%)
125	98 ± 1.34
250	95 ± 2.54
500	92 ± 2.88
1000	87 ± 5.38

free amino group plays a pivotal role in the free radical scavenging activity. Moreover, AEC significantly quenched hydroxyl radical compared to other three radicals at low concentration. Cacciuttolo et al.[21] reported that the chemical activity of hydroxyl radical in various reactive oxygen species is the strongest, which can easily react with biomolecules such as amino acids, proteins, and DNA. Therefore, the removal of hydroxyl radical is probably one of the most effective defense of a living body against various diseases.

Water-soluble chitin derivative was successfully prepared by chemical modification to introduce amino functionality on C-6 position. AEC was investigated for antioxidant activity using the ESR spin-trapping technique. The results revealed that AEC had obvious antioxidant potential due to the free amino group. Especially, AEC showed more potent radical scavenging activity against hydroxyl radical.

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