Original Article

Pseudo flow-injection ESR technique combining spin-trapping and application to the evaluation of superoxide scavenging capacity of phenolic compound

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Abstract

Evaluation methods of superoxide scavenging capacity have been studied by many researchers. One of the methods is a spin-trapping electron spin resonance (ESR) technique, in which 5,5-diemthyl-1-pyrroline 1-oxide (DMPO) was usually used as a spin-trapping reagent. In previous reports, the evaluation method based on a competition reaction and spin-trapping ESR technique were proposed. However, a big key issue and unavoidable problem remained in this evaluation. This is the rapid decrease of DMPO-O_2^- adduct by the following chemical reactions. Therefore, the initial signal intensity of DMPO-O_2^- has to be estimated with an approximation from a decreasing curve. This approximation, however, includes at least a thirty-second time lag before starting an ESR measurement. So, the measurement of a shorter time lag is required to obtain a more accurate initial intensity of DMPO-O_2^- adduct. In this study, the pseudo flow-injection ESR method using a peristaltic pump was adopted to inject the test solution directly into a quartz cell in ESR cavity. This method achieved a 10 times shorter time lag (ca. 3 seconds) compared with the previous method (ca. 30 seconds) using a hematocrit capillary. As a consequence, the initial signal intensity within 10 % error was obtained without any estimation. The second-order rate constants of the reaction between various phenolic compounds and superoxide were obtained as one of the applications of this method.

Key words

spin-trapping ESR, electrochemically-generated superoxide, competition reaction, superoxide scavenging capacity, phenolic compound

1. Introduction

It is well known that reactive oxygen species (ROS) have been the initiator of oxidative stress, aging, inflammation, immunological disorder, and other certain chronic diseases (Koide et al., 2000; Liu et al., 2001; Petersen et al., 2005; Tarpey and Fridovich, 2001). Therefore, the evaluation methods of ROS scavenging capacity have been studied by many researchers (Koide et al., 2000; Liu et al., 2001; Ou et al., 2001; Sueishi et al., 2010). Since ROS is of a very short-life species, more sensitive techniques are required. Spin-trapping ESR is the most useful analytical method permitting the direct detection of ROS.

For examples of useful methods, oxygen radical absorbance capacity (ORAC) that was based on fluorescent and ESR was called $ORAC_{FL}$ (Ou et al., 2001) and $ORAC_{ESR}$ (Sueishi et al., 2010), respectively. Until 2012, ORAC assay was one of the often-employed methods for ROS scavenging capacity (Niki, 2012; USDA's NDL home page). Now, evaluation methods that can be used as an official method are much needed. However, there is no ROS scavenging capacity evaluation method as an official method.

For the evaluation, superoxide ($O_2^{\bullet-}$) generating systems were important and over 12 systems have been reported by many researchers (Clément et al., 2005; Finkelstein et al., 1979; Hatano et al., 1989; Kawai et al., 2011; Kładna et al., 2013; Koide et al., 2000; Liu et al., 2001; Maricle and Hodgson, 1965; Pieta et al., 2008; Pou et al., 1990; Rosen and Turner III, 1988; Tarpey and Fridovich, 2001; Ueno et al., 1984; Ukeda, 2004). Especially, photolysis (Kawai et al., 2011), potassium superoxide (KO_2) and 18-crown-6 ether (Kładna et al., 2013; Rosen and Turner III, 1988; Ueno et al., 1984), electrolysis of molecular oxygen (Liu et al., 2001; Maricle and Hodgson, 1965; Pieta et al., 2008), and xanthine- (or hypoxanthine)-xanthine oxidase system (XAN-XOD or HPX-XOD)) (Clément et al., 2005; Finkelstein et al., 1979; Koide et al., 2000; Pou et al., 1990; Rosen and Turner III, 1988; Ueno et al., 1984) were often used to evaluate superoxide scavenging capacity by ESR spin-trapping.

In the past, superoxide scavenging capacity evaluation method based on competition reaction and ESR spin-trapping technique was reported (Koide et al., 2000; Liu et al., 2001). This method makes it possible to evaluate the second-order rate constants or IC_{50} of antioxidant compounds, in which an electrochemically generated superoxide was used as one of the best sources of superoxide.

In this paper, the rapid evaluation method of superoxide

scavenging capacity that was enhanced by using a peristaltic pump like a flow-ESR technique (Kawai et al., 2011), or flow-injection ESR (FI-ESR) system (Komatsu-Watanabe et al., 2008) is proposed. As the applications of this system, the second-order rate constants of the reactions of superoxide and several phenolic compounds are determined.

2. Experimental

2.1 Chemicals

Spin-trapping reagent, 5,5-diemthyl-1-pyrroline 1-oxide (DMPO, 8.7 M, M = mol dm^{-3}) was obtained from Labotec, Japan. Anhydrous dimethyl sulfoxide (DMSO), phosphate buffer powder (1/15 M, pH 7.4), tetraethylammonium perchlorate (TEAP), rutin, catechol, quercetin (Que), catechin, pyrogallol (Py), myricetin (My), 3.4-dihydroxy toluene (3,4-DHT), hydroquinone (HQ), and ethanol (EtOH) were purchased from Wako Pure Chemical Industries, Japan. Chlorogenic acid (CA), gallic acid (GA), 1,2,4-trihydroxybenzene (1,2,4-THB) were obtained from Tokyo Chemical Industry, Japan. Oxygen gas was from Yamagata Sanso, Japan.

A phosphate buffer solution (PBS) was prepared from phosphate buffer powder at a concentration of 0.1 M. TEAP dried in vacuo at 60 °C for a few hours. DMSO was dewatered by recrystallization just before use. Eighty percent of the EtOH solution was prepared using EtOH and PBS (80/20, v/v). The other reagents were used without further purification.

2.2 Electrolytic cell and superoxide generation

The electrolysis cell consisted of a glassy carbon disc electrode (GC-10), coiled platinum wire, and coiled silver wire, which were used as a working electrode (WE), a counter electrode (CE), and a reference electrode (RE), respectively. In a screw vial, CE and RE were separated by a glass tube with sintered glass.

The electrolyte of TEAP was dissolved in refined DMSO at a concentration of 50 mM. Using this solution, electrolysis was carried out under potentiostatic condition at -0.75 V vs. Ag (RE) over 10 minutes with O_2 bubbling by using potentiostat/galvanostat HA-211 (Hokuto Electronics, Japan). Then, the galvanostatic electrolysis was carried out to generate continuously superoxide at a controlled current of -0.4 mA under an overflow of O_2 gas. In this condition, superoxide existed stably for over 3 hours, and its concentration was estimated to be over 0.1 mM by the ESR spectrum of DMPO- O_2^-.

2.3 ESR measurement and superoxide scavenging capacity evaluation

Our previous study was as follows. In a micro test tube (1.5 mL size), 200 µL of 0.1 M PBS (i.e. scavenger free soln.), 20 µL of 8.7 M DMPO, and 50 µL of electrogenerated superoxide / DMSO solution were added and mixed using a magnetic stirrer CT-1 (As One, Japan). This mixture solution was taken into a hematocrit capillary (Terumo, Japan), and the end of the capillary was sealed with putty. As shown in Figure 1 (a), the selected ESR signal from 30 to 78 seconds was recorded by auto sampling mode at a 12 second interval. Then, the time course of the signal height was obtained, the initial signal intensity, namely, signal height just after mixing, was estimated with exponential approximation. The results are indicated in Figure 1 (a). However, about a 30 second time lag could not be avoided, even with expert experimentation.

To overcome this problem, the same mixture solution was injected quickly into a quartz cell set in an ESR cavity via a peristaltic pump PST-100 (IWAKI, Japan) at the speed of 0.036 mL/sec. The time course of the signal intensity was recorded under the ESR condition of the magnetic field adjusted at the maximum of the selected signal. After about 3 seconds from injection, the test solution was reached in a quartz cell. Soon after an ESR signal appeared, the peristaltic pump was stopped immediately. The decay of ESR signal intensity was recorded for 10 seconds. This method is called "pseudo flow-injection ESR" as shown in Figure 1 (b). All ESR measurements were carried out with an X-band ESR spectrometer JES-FR30 (JEOL, Japan).

All phenolic compounds illustrated in Figure 1 (c) were dissolved in PBS or 80 % EtOH and diluted by PBS at a suitable concentration for superoxide scavenging capacity evaluation. The capacities were evaluated from the following equation (1) based on competition reaction (Figure 2) (Koide et al., 2000).

$$I_0 / I - 1 = (k_{Scavenger}\,[Scavenger]) / (k_{DMPO}\,[DMPO]) \quad (1)$$

In equation (1), I_0 and I indicate DMPO-O_2^- signal intensity in the absence of scavenger and in the presence of scavenger, respectively. [Scavenger] and [DMPO] mean the final concentration of scavenger and DMPO, respectively.

At first, IC_{50} was calculated from the calibration curve of phenolic compounds. This curve means the relationship between $I_0 / I - 1$ and the concentration of phenolic compound. The IC_{50} is the concentration at $I_0 / I - 1 = 1$. That is, IC_{50} means the concentration indicating half signal intensity against the signal intensity of the solution without any antioxidant. After the calculation of IC_{50}, the second-order rate constant ($k_{Scavenger}$) of the reaction between phenolic compound and superoxide was determined using $I_0 / I - 1 = 1$, [Scavenger] = IC_{50}, and [DMPO] = 0.6444 M. Equation (1) was redefined as equation (2).

$$k_{Scavenger} = k_{DMPO} * (0.6444) / IC_{50} \quad (2)$$

However, the second-order rate constant of DMPO (k_{DMPO}) with superoxide at pH = 7.4 was reported to be 30 [$M^{-1}s^{-1}$], (Koide et al., 2000) and 2.4 [$M^{-1}s^{-1}$] (Keszler et al., 2003). In this study, $k_{Scavenger}$ was calculated using k_{DMPO} = 30 [$M^{-1}s^{-1}$].

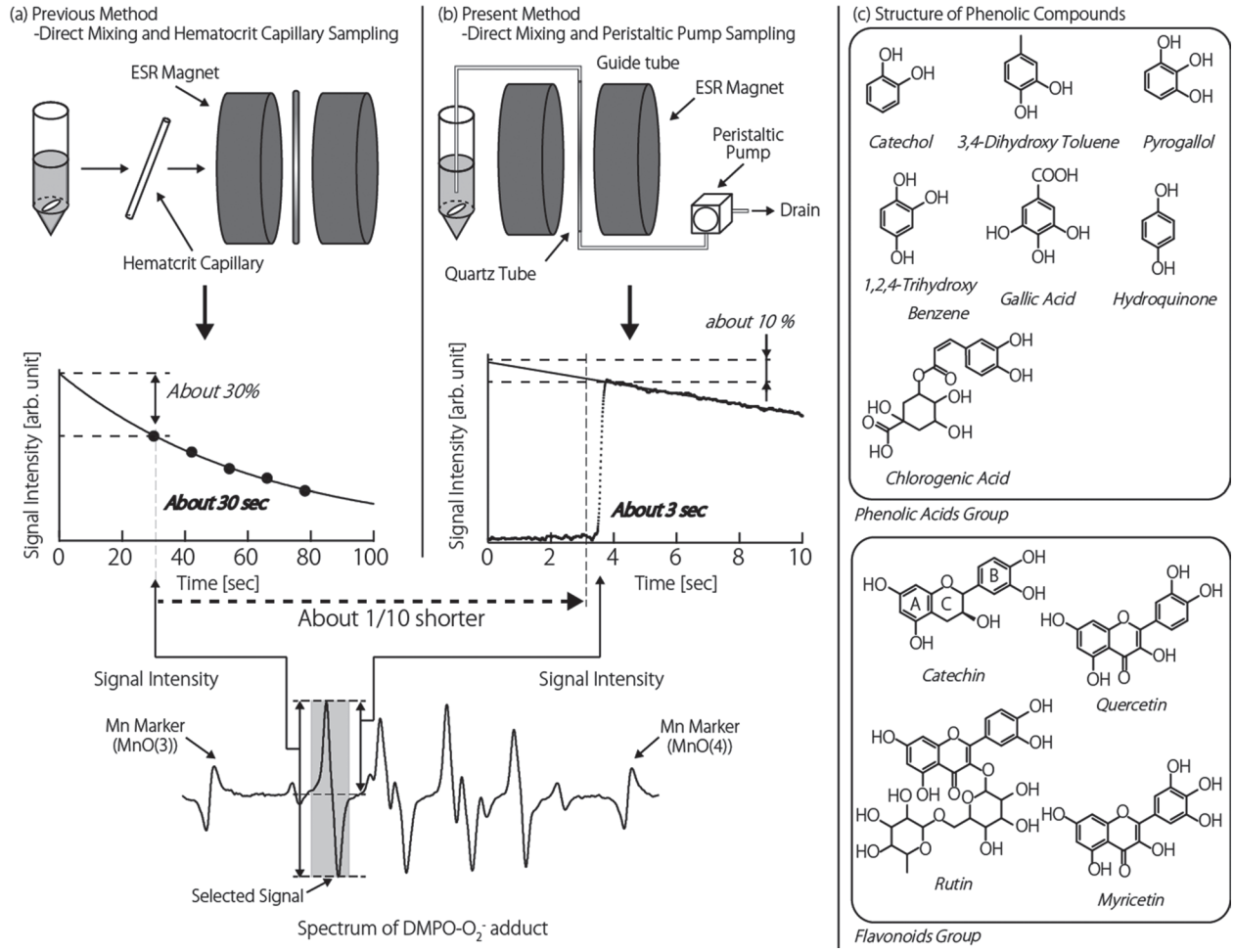

Figure 1: The spectrum of DMPO-O_2^- adduct and decay curves of DMPO-O_2^- adduct at selected signal (dark colored)

Note: Superoxide was prepared by electrolysis of molecular oxygen in 50 mM TEAP/DMSO solution. Test solution was made from 200 µL of PBS (i.e. scavenger free solution), 20 µL of DMPO, and 50 µL of electrochemically generated superoxide in a micro test tube. In this figure, two methods are illustrated. Figure 1(a) is a manual sampling procedure (Previous method), and Figure 1(b) is a direct injection into quartz capillary cell in ESR cavity via a peristaltic pump. (Pseudo flow-injection ESR, Present method). In Figure 1 (c), the structures of phenolic compounds used here are indicated.

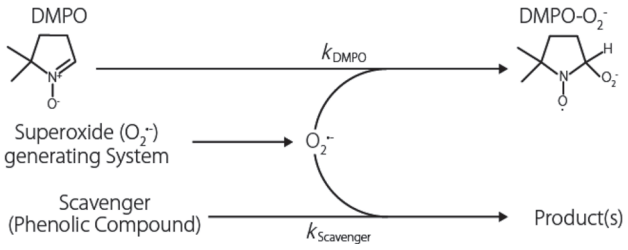

Figure 2: Outline of competition reaction

3. Results and Discussion

3.1 Ability of pseudo flow-injection ESR method

As mentioned above, stable superoxide generation was accomplished by one-electron reduction of molecular oxygen using 5 mL of 50 mM TEAP/DMSO solution over 3 hours. However, it is also very important to estimate the initial signal intensity of DMPO-O_2^-. In a previous experiment, the initial signal intensity of DMPO-O_2^- was estimated with an exponential approximation of the decay curve of DMPO-O_2^-. However, this method had a 30 second time lag as described above. Therefore, a large error would be included in the calculated initial intensity.

In the direct injection, namely, pseudo FI-ESR, the ESR signal intensity increased in just about 3 seconds after mixing the test solution, and the maximum of signal intensity, which appeared at around 4 seconds, had fewer losses (almost less than 10 %). This improved method drastically decreased (almost 10 times shorter) the time lag to measure the ESR signal intensity (Figure 1 (b)). As a consequence, the pseudo FI-ESR technique combining spin-trapping is thought to be useful and effective in the evaluation of superoxide scavenging activities.

3.2 Superoxide scavenging capacity of phenolic compounds

Superoxide scavenging capacities of phenolic compounds were evaluated from calibration curves shown in Figure 3.

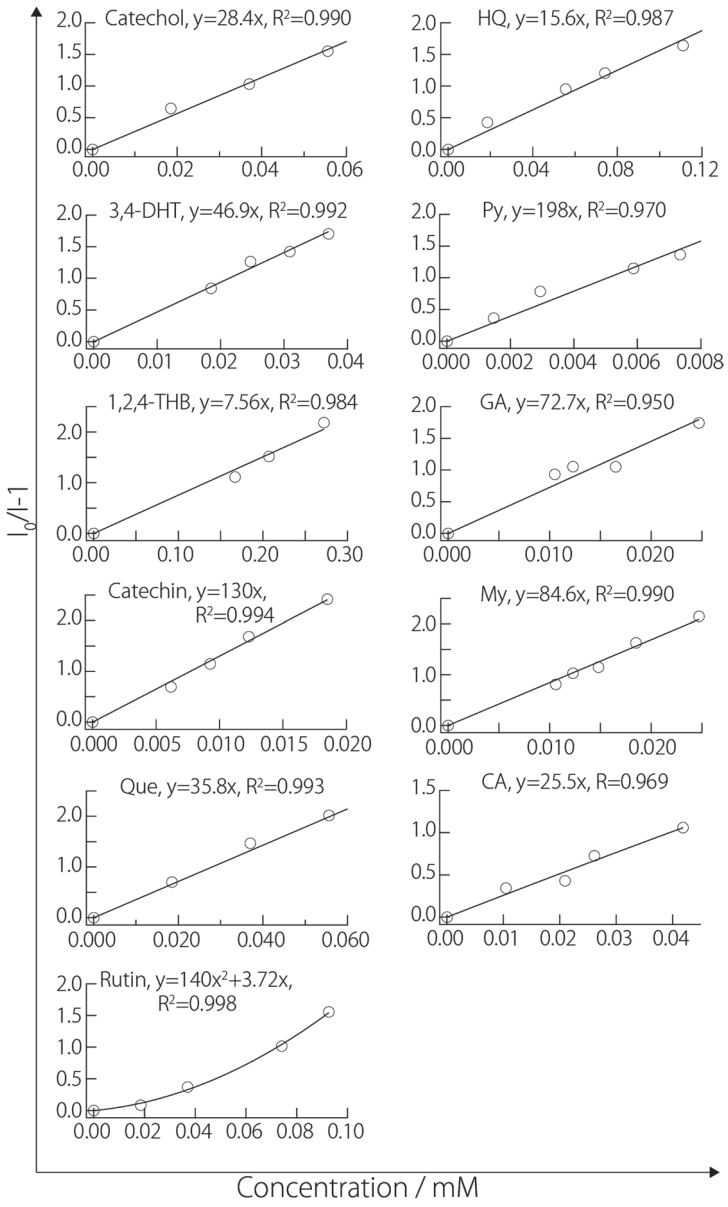

Figure 3: Calibration curve of several phenolic compounds

Note: IC_{50} in Table 1 were calculated at $I_0 / I - 1 = 1$ in these calibration curves.

From the curve, IC_{50} was firstly obtained and then $k_{Scavenger}$ was calculated from equation (2). The results are summarized in Table 1.

Phenolic compounds used here were divided into two groups; i.e. "Phenolic Acids" and "Flavonoids". Catechol, HQ, 3,4-DHT, Py, 1,2,4-THB, GA and CA were categorized into "Phenolic Acids". Catechin, My, Que, and rutin were categorized into "Flavonoids".

In the case of phenolic compounds containing 1,2-dihydroxy benzene (catechol) structure, catechin indicated the highest superoxide scavenging capacity. 3,4-DHT, Que, catechol, CA, rutin and 1,2,4-THB gave a superoxide scavenging capacity lower than catechin. Rutin (aglycone of Que and β-rutinose) was 2.6 times lower than Que. 3,4-DHT had 6.2 times higher superoxide scavenging capacity against 1,2,4-THB. 1,2,4-THB had the lowest superoxide scavenging capacity of all phenolic compounds.

On the other hand, the phenolic compounds containing 1,2,3-trihydroxy benzene (pyrogallol) structure showed stronger superoxide scavenging capacity than that of phenolic compounds containing catechol structure except catechin. Py had the highest superoxide scavenging capacity of all phenolic compounds tested in this study.

Second-order rate constants ($k_{Scavenger}$) of several phenolic compounds evaluated in this work, have already been reported by other researchers. (Jovanovic et al., 1994; Kitagawa et al., 1992; Sakurai, 2014; Sueishi et al., 2014; Tanbert et al., 2003) Considering the amazingly short lifetime of superoxide

Table 1: IC_{50} and second-order rate constants ($k_{Scavenger}$) of typical phenolic compounds

Phenolic compounds	MW (g/mol)	IC_{50} (μM)	$k_{Scavenger} \times 10^5$ (M^{-1}•s^{-1})*1					
			Present method	Ref. 1	Ref. 2	Ref. 3	Ref. 4	Ref. 5
Catechol	110.1	35.2	5.49	29.6				
HQ	110.1	64.1	3.02	1.15				
3,4-DHT	124.1	21.3	9.07					
Py	126.1	5.05	38.3	222				
1,2,4-THB	126.1	132	1.46					
GA	170.1	13.8	14.1	205		4.96	0.180	2.88
Catechin	290.3	7.69	25.1			1.29		
My	318.2	11.8	16.4			5.52	0.470	
Que	338.3	27.9	6.92	117		1.07		
CA	354.3	39.2	4.39		9.06		0.510	
Rutin	610.5	72.3	2.67		5.61	2.51	0.180	0.154

Notes:
Annotation: HQ, 3,4-DHT, Py, 1,2,4-THB, GA, My, Que, and CA mean hydroquinone, 3,4-dihydroxy toluene, pyrogallol, 1,2,4-trihydroxy benzene, gallic acid, myricetin, quercetin, and chlorogenic acid, respectively. MW means molecular weight.
*1 $k_{Scavenger}$ was calculated from IC_{50} using k_{DMPO} = 30 $M^{-1}s^{-1}$ (Present method, at pH = 7.4), and other experimental conditions in references are summarized below. (Ref. No.: Author; Superoxide generating system, Detection method or apparatus, k_{DMPO})
Ref. 1: Kitagawa et al., 1992; HPX-XOD system, Spin-trapping ESR using DMPO, k_{DMPO} = 50 $M^{-1}s^{-1}$ (Villamena and Zweier (2002) at pH = 7.0)
Ref. 2: Sakurai et al., 2014; Riboflavin and UV-Light irradiation at pH = 7.4, Spin-trapping ESR using DMPO, k_{DMPO} = 30 $M^{-1}s^{-1}$
Ref. 3: Tanbert et al., 2003; KO_2 and dicyclohexyl-18-crown-6 ether system at pH = 7.4, Chemiluminescence, k_{DMPO} = 30 $M^{-1}s^{-1}$. $k_{Scavenger}$ was calculated using equation (2) from the value of IC_{50} in the paper.
Ref. 4: Jovanovic et al., 1994; γ radiolysis in aqueous solution at pH = 10, 3-MeV van de Graaff pulse radiolysis apparatus, $k_{Scavenger}$ was the original value in the paper.
Ref. 5: Sueishi et al., 2014; Riboflavin and UV-Light irradiation at pH = 7.4, Spin-trapping ESR using CYPMPO, $k_{Scavenger}$ was the original value in the paper.

in aqueous media and the difference of experimental procedure (i.e. pH, apparatus, detection method, and spin-trapping reagent), the differences of $k_{Scavenger}$ values in Table 1 may be acceptable except the values of Ref. 4. In this paper, pH was adjusted to be about 10. In other references pH 7 was used. These results suggest that pH control is very important in the evaluation of the superoxide scavenging ability for phenolic compounds.

4. Conclusions

The evaluation of superoxide scavenging capacity was demonstrated using a pseudo flow-injection ESR technique and superoxide generated electrochemically with molecular oxygen dissolved in DMSO containing 50 mM of TEAP as an electrolyte. Using the pseudo flow-injection ESR, the time lag to start ESR measurement was decreased extremely by about 10 times (about 30 to 3 seconds) than the usual ESR method. The values of $k_{Scavenger}$ obtained in this study were shown to be acceptable values from the comparison of $k_{Scavenger}$ reported by other researchers.

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