SHORT COMMUNICATION

Reactive oxygen radical-scavenging activity assay by photolysis of azo-radical initiator without exposing samples to ultraviolet light: A preliminary feasibility study of a modified assay

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Summary

We previously developed an electron paramagnetic resonance (EPR)-based oxygen radical absorbance capacity (ORAC-EPR) assay that can be used to evaluate the radical-scavenging activity (RSA) of samples by employing the established EPR spin trap method. In this assay, since reactive oxygen radicals are generated by the photolysis of the azo-radical initiator by ultraviolet (UV) light irradiation, the sample in the assay mixture exposed to UV light may also get photolysed. In the present study, irradiation of the sample was avoided; a novel radical-generation method was adopted in the ORAC-EPR assay, and the feasibility of the modified assay was assessed. RSA was evaluated in the modified as well as in the conventional assay with three antioxidants. Although the RSA values normalized to the spin trap reagent obtained with the modified assay were approximately 20% lower than those obtained with the conventional assay, the maximum difference between the RSA values of the two methods was 15% when this data were normalized to the standard antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Elucidation of the generation mechanism of the oxygen radicals and the reasons for lower RSA is necessary prior to application of the new radical-generation method coupled to ORAC-EPR assay.

Keywords

radical-scavenging activity; 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH); ultraviolet light; electron paramagnetic resonance

In a previous study, we proposed a rapid and simple radical-scavenging activity (RSA) assay named as electron paramagnetic resonance (EPR)-based oxygen radical absorbance capacity (ORAC-EPR) assay that uses the EPR spin trap method [1]. In that assay, the alkoxyl and peroxyl radicals, that are reactive oxygen-centred radicals with high oxidizing power, were generated by the photolysis of an azo-radical initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) through irradiation with UV light (Eq. 1, 2; R = $HCl \bullet HN(H_2N)C(CH_3)_2C$) [2–4].

$$AAPH + 2O_2 \xrightarrow{UV} 2ROO^{\bullet} + N_2 \quad (1)$$

$$2ROO^{\bullet} \rightarrow 2RO^{\bullet} + O_2 \quad (2)$$

The time required for the assay was reduced to 60 s, while the conventional assay using AAPH thermolysis requires 35 min [5]. The generated alkoxyl radicals, captured by the spin trap reagent, were quantified by EPR spectrometry as spin adducts in the presence and absence of antioxidants. The relative RSA value of the sample to the spin trap reagent was obtained by employing a simple equation based on the theory of competing reactions. Because the examined sample, coexisting with AAPH in the assay mixture, was also exposed to UV light, photochemical reactions that can sometimes lead to RSA deterioration can be triggered when the sample is UV-sensitive [6].

Recently, we reported that the reactive oxygen radicals from cooled AAPH were generated even after the termination of UV irradiation [7]. It was believed that the radicals were generated from the unstable *cis*-configured AAPH, to which the stable *trans*-configured AAPH was isomerized by the UV

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light irradiation, and remained even after termination of irradiation because of a decrease in the decomposition rate at low temperatures [8]. The purpose of this preliminary study was to examine the feasibility of the modified ORAC-EPR assay by applying a new radical-generation method. The RSA of chemicals were evaluated by the modified assay, and the results were compared with those obtained by using the conventional ORAC-EPR assay.

MATERIALS AND METHODS

AAPH, caffeic acid, *n*-propyl gallate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and sodium phosphate were purchased from Wako Pure Chemical Industries (Osaka, Japan). The spin trap reagent CYPMPO (5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide) was obtained from Radical Research (Tokyo, Japan). All chemicals, except sodium phosphate, were dissolved in 100 mmol·l^{-1} phosphate buffer (pH 7.4).

Instrumentation

The UV light irradiator RUVF-203SR (Radical Research) was equipped with a mercury-xenon lamp (200 W; San-ei Electronics, Osaka, Japan), which was used as a light source. For EPR measurements, the RRX-1XS X-band ESR spectrometer (JEOL, Tokyo, Japan) and the WIN-RAD operation software (Radical Research) were used. The spectrometer settings were as follows: field modulation frequency, 100 kHz; field modulation width, 0.1 mT; microwave power, 6 mW; field scan width/rate, ± 7.5 mT/2 min; and time constant, 0.1 s. A disposable borosilicate flat cell (Radical Research) loaded with the assay mixture was placed in the EPR cavity and the EPR signal was recorded. The hyperfine coupling constants (HFCCs, A(H), A(N), A(P)) were estimated by fitting the Gaussian-type function to the observed spectrum by using the least squares method. Because the CYPMPO peroxyl radical adduct had a shorter half-life (4.4 s) than the CYPMPO alkoxyl radical adduct (613 min), the EPR spectrum of the stable alkoxyl radical adduct was recorded in this study [9].

Conventional ORAC-EPR assay at room and low temperatures

RSA values of caffeic acid, *n*-propyl gallate and Trolox were evaluated by using the conventional and modified assay method. In the assay at room temperature, an assay mixture of 10 mmol·l^{-1} AAPH, 10 mmol·l^{-1} CYPMPO and 0.1 mmol·l^{-1} antioxidant solutions was loaded in the flat cell placed in the EPR cavity. The assay mixture was irradiated with UV light, whose power was attenuated to minimize photochemical damage of the sample, for 60 s and the EPR signal was recorded. In the assay at low temperature, the assay mixture of 0.45 mmol·l^{-1} AAPH, 10 mmol·l^{-1} CYPMPO and 50 μmol·l^{-1} antioxidant (caffeic acid, Trolox) or 10 μmol·l^{-1} antioxidant (*n*-propyl gallate) was irradiated with UV light for 6 s while being cooled in a salt ice bath (< –10°C), and the EPR signal was recorded.

Modified ORAC-EPR assay

Briefly, 54 μl of a 1 mmol·l^{-1} AAPH solution was irradiated with UV light for 60 s while being cooled in a salt ice bath (< –10°C). Then, 3 μl of a 200 mmol·l^{-1} CYPMPO solution and 3 μl of the sample solution were mixed and added to the AAPH solution at 5 s after termination of the irradiation. The EPR signal of the assay mixture was recorded. The interval between termination of the irradiation and addition of the mixed solution was chosen to ensure reproducibility of the manipulation. The final concentration of the antioxidants was 50 μmol·l^{-1} (caffeic acid and trolox) or 10 μmol·l^{-1} (*n*-propyl gallate). RSA was evaluated once a day on 2 days (Day 1 and Day 2).

Data analysis for RSA evaluation

RSA was evaluated by the normalized rate constant. The details of kinetic formulation were published elsewhere [10]. Finally, the rate constant of the antioxidant (A) normalized by the spin trap CYPMPO (C) was as follows:

$$\frac{k_A}{k_C} = \frac{I_B - I}{I} \cdot \frac{[C]_0}{[A]_0} \quad (3)$$

I_B denotes the height of the fifth peak from the lower-magnetic-field side in the presence of the spin trap alone, I denotes the height of the same peak in the combined presence of the antioxidant and spin trap, and $[A]_0$ and $[C]_0$ denote the initial concentrations.

The peak height was considered to be proportional to the concentration of the spin adduct in the assays. Recently, RSA of the sample towards the peroxyl radical generated by photolysis of AAPH was measured by using the EPR spin trap method [9]. Because the peroxyl radical is scavenged by the sample in the presence of antioxidants, the amount of the generated alkoxyl radical adduct is also decreased because of a decrease in the alkoxyl-radical generation as well as scavenging of the alkoxyl radical (Eq. 2). There-

fore, the measured RSA in our assays was considered to include the combined RSA of the sample towards both the alkoxyl and peroxyl radicals.

RESULTS AND DISCUSSION

The EPR spectrum obtained by the modified ORAC-EPR assay is shown in Fig. 1. The HFCCs were estimated as follows: A(H) = 1.21 mT, A(N) = 1.36 mT, A(P) = 4.77 mT. These values were almost the same as those reported previously [11]. It was confirmed that the same alkoxyl radical was generated.

The RSA values obtained by using the modified and conventional ORAC-EPR assays are summarized in Tab. 1. In the modified assay, the difference between the RSA normalized to spin trap reagent on the two days was less than 10%. The RSA values obtained by using the modified assay were by approx. 20% lower than those obtained by the conventional assay. Because CYPMPO and the sample were added simultaneously to the AAPH solution, same conditions existed for the competing reactions of CYPMPO and the sample with the radicals in the modified assay. Because the results obtained by the conventional assay at a low temperature were not as low as those obtained by the modified assay, the low temperature was not responsible for the lower RSA values in the modified assay. When the RSA data were normalized to Trolox, the difference between the results of the modified and conventional assays was reduced to not more than 15%. Considering this fact, the lower RSA values can be believed to have originated from CYPMPO, the spin adduct, or the experimental conditions unique to the modified assay, rather than from a mutually unrelated decrease in RSA of each antioxidant. Here, two possible causes of the lower RSA are shown. One possibility is overestimation of RSA in the conventional assay due to photodamage of CYPMPO by UV light. Another possibility is underestimation of RSA in the modified assay due to the concentration of CYPMPO higher than that of the antioxidant. It was believed that the distribution of CYPMPO was faster than that of the antioxidants because of the larger flux of CYPMPO at the time of the addition of CYPMPO and the antioxidant to the AAPH solution. In various RSA assays, RSA of the samples are often represented after normalization to Trolox [5, 12, 13]. Thus, the modified ORAC-EPR assay could be suitable when the RSA values are normalized to Trolox. However, evaluation of more antioxidants and a comparison of the results to the conventional assay is needed to confirm the possibility.

Although the same alkoxyl radicals were considered to be generated by the modified method, direct observation of the *cis*-isomer is necessary to elucidate the radical generation mechanism after UV irradiation. Elucidation of the behaviour of the *cis*-isomer would also facilitate optimization of the parameters used to generate the oxygen radical.

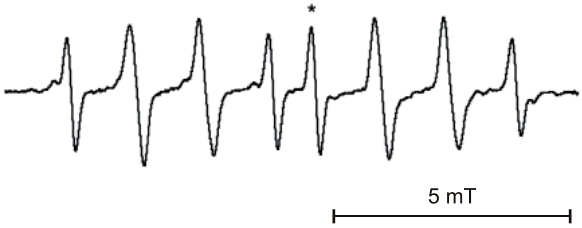

Fig. 1. Averaged electron paramagnetic resonance spectrum of five spectra observed in the modified assay.

The height of the peak marked with an asterisk was used in the data analysis.

Tab. 1. Summary of radical-scavenging activity obtained by modified and conventional electron paramagnetic resonance-based oxygen radical absorbance capacity assays.

Assay	RSA normalized to spin trap reagent (k_A/k_C)					RSA normalized to Trolox		
	Modified			Conventional		Modified	Conventional	
	Day 1	Day 2	Average*	Room temperature	Low temperature		Room temperature	Low temperature
Trolox	160 ± 15	170 ± 54	170 ± 28	220 ± 21	230 ± 24	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
Caffeic acid	370 ± 55	340 ± 35	360 ± 32	430 ± 28	410 ± 57	2.2 ± 0.4	2.0 ± 0.2	1.8 ± 0.3
n-Propyl gallate	550 ± 57	590 ± 110	570 ± 61	680 ± 42	990 ± 200	3.5 ± 0.7	3.0 ± 0.3	4.4 ± 1.0

RSA – radical-scavenging activity. Values of RSA normalized to spin trap reagent are given as average ± standard deviation (n = 5). Values of RSA normalized to Trolox are given as ratio to Trolox ± propagated error to the ratio.
* – average of Day 1 and Day 2 ± propagated error to the average.

CONCLUSIONS

Elucidation of the generation mechanism of the oxygen radicals and lower RSA are required to apply the new radical generation method to the ORAC-EPR assay.

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