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Cyclodextrin Inclusion Interferes with Trolox Oxygen Radical Scavenging Capacity Measurement

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The interference of cyclodextrin solubilization with the measurement of oxygen radical scavenging capacity was investigated. Cyclodextrin (CD) that can solubilize water-insoluble compounds has been used in the oxygen radical scavenging capacity assay called oxygen radical absorbance capacity (ORAC) method. A vitamin E analog, trolox (2-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) has been employed as a standard compound in ORAC methods and the results were often expressed in the trolox equivalent unit. We found that trolox ORAC values measured with electron spin resonance-based ORAC method, were markedly dependent on the CD concentration, *i.e.*, it decreased by 50% when [CD]/[Trolox] = 100 was present. 2D ROESY NMR study of trolox/CD inclusion complex revealed that trolox resided within the CD cavity. The reactive phenoxyl group in trolox is shielded from the attack by the oxygen radical, suggesting that this hindrance was causal for the decrease in ORAC values.

1. Introduction

Evaluations of antioxidant capacity of pure antioxidants and food extracts have been performed by using the fluorescence-based oxygen radical absorbance capacity method (ORAC-FL) [1–3]. In order to overcome some experimental disadvantages of ORAC-FL, such as the necessity of time-course measurements and computer-aided analysis, we have proposed a new method of ORAC evaluation using electron spin resonance (ESR) spin trapping technique (ORAC-EPR or ORAC-ESR) [4,5]. The ORAC-ESR method, in which the free radicals are directly quantified with ESR spin trapping, is a simple and useful method for the radical scavenging rate determination of antioxidants. Because natural antioxidants are often lipophilic and water-insoluble, Huang *et al.* adopted a cyclodextrin (CD) analog to solubilize lipophilic compounds [6–8]. CD

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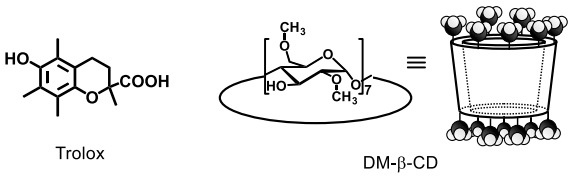

Fig. 1. Structures of antioxidant trolox and modified β-cyclodextrin (DM-β-CD).

molecule is a cyclic oligomer of glucose, having a cage-shaped void space, wherein a lipophilic molecule is included and solubilized [9,10]. However, possible interference between the ORAC assay and the solubilizer is not well understood.

In most ORAC-like methods, a vitamin E analog, 2-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox, Fig. 1) has been employed as a standard antioxidant of the radical scavenging capacity. Thus, ORAC values are often expressed in the trolox equivalent unit. Fortunately, trolox is slightly water soluble and its ORAC value can be evaluated in the absence or presence of solubilizers. When a cyclodextrin analog, DM-β-CD (2,6-*di*-*O*-methylated β-cyclodextrin, Fig. 1) was added to the trolox solution, ORAC values markedly decreased as a function of increasing CD concentration. In order to study the cause of this decrease, trolox/DM-β-CD complex was characterized with 2D ROESY NMR. The structure has lead us to the conclusion that trolox's reactive site was effectively included in the CD cavity, blocking the approach of the oxygen radical, which resulted in the decrease of the scavenging rate.

2. Experimental

2.1 Materials

Chemical structures of the compounds used in this study are shown in Figs. 1 and 2. Trolox and DM-β-CD were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and Nakalai Tesque (Kyoto, Japan), respectively. The spin-trap DMPO (5,5-dimethyl-pyrroline *N*-oxide) was obtained also from Tokyo Chemical Industry Co. (Tokyo, Japan). As a source of oxygen radical (alkoxyl radical (RO·)), 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) was purchased from Wako Pure Chemicals (Osaka, Japan) (Fig. 2).

2.2 ESR and NMR measurements

A competitive reaction method between the spin trap DMPO and trolox was employed to quantify the radical scavenging capacity (ORAC values) [4,5]. A phosphate buffer (0.1 M (M = mol dm^{-3}), pH = 7.4) was used as a solvent and the RO· radical was generated with *in situ* UV irradiation (1 s irradiation time with a RUF-203S 200 W mercury arc (Radical Research Inc.)). The ESR signals of RO· radical adducts with DMPO were recorded in a JEOL FA200 X-band spectrometer (Akishima, Japan). The sample solution temperature was controlled at 298 ± 0.1 K.

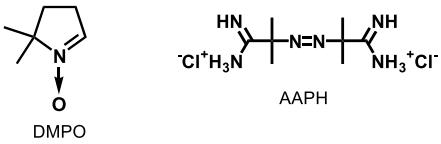

Fig. 2. Structures of spin trap (DMPO) and radical source (AAPH).

In order to elucidate the structure of the DM-β-CD inclusion complex, ^{1}H-NMR spectra were obtained in D_2O with a Varian Mercury 300 NMR spectrometer (300 MHz) (Palo Alto CA, USA) at room temperature. Chemical shifts were reported as δ values relative to HOD (δ 4.79) as an internal standard [11]. A 2D ROESY NMR experiment was performed at 600 MHz in D_2O on a Varian VNS600 NMR spectrometer at 303 K. The mixing time for the ROESY NMR experiments was set at 200 ms.

2.3 Scavenging capacity measurement

In the present system, trolox and DMPO compete to scavenge AAPH-derived oxygen radical (RO· radical). Thus, the scavenging capacity of trolox was obtained relative to that of DMPO [4,5]. The reaction that we monitored in excess of CD is illustrated as follows [12]:

$$\mathrm{AAPH} \xrightarrow{h\nu} \mathrm{RO}\cdot$$

$$\mathrm{DMPO:CD} + \mathrm{RO}\cdot \longrightarrow \mathrm{DMPO\text{-}OR:CD} \qquad \text{scavenging rate const: } k_{\mathrm{DMPO}}$$

$$\mathrm{Trolox:CD} + \mathrm{RO}\cdot \longrightarrow \mathrm{Product\ (ESR\ silent)} \qquad \text{scavenging rate const: } k_{\mathrm{Trolox}}\ ,$$

where DMPO : CD and Trolox : CD denote CD-inclusion complexes of DMPO and trolox, respectively. A simple formulation for the radical scavenging calculation can be derived from the above reaction scheme and has been reported elsewhere [4,5]. The resultant equation is:

$$\frac{I_0 - I}{I} = \frac{k_{\mathrm{Trolox}}}{k_{\mathrm{DMPO}}} \frac{[\mathrm{Trolox}]_0}{[\mathrm{DMPO}]_0}\ , \tag{1}$$

where I and I_0 are ESR signal heights for the radical adduct in the presence and absence of trolox, respectively, and the $[\ ]_0$ symbol denotes the initial concentrations of DMPO or trolox. The measurement of ESR signal intensities by selecting several trolox concentrations would give a linear plot of $(I_0 - I)/I$ against $[\mathrm{Trolox}]_0/[\mathrm{DMPO}]_0$ like Fig. 3c. The slope of such a plot yielded $k_{\mathrm{Trolox}}/k_{\mathrm{DMPO}}$, which is equivalent to the radical scavenging rate constant for trolox relative to that of DMPO.

3. Results

3.1 ESR spectrum of spin-trapped oxygen radical

Figure 3a shows the ESR spectra of RO· radical spin-trapped with DMPO in a UV-irradiated AAPH solution in the absence of DM-β-CD. The hyperfine splitting constant

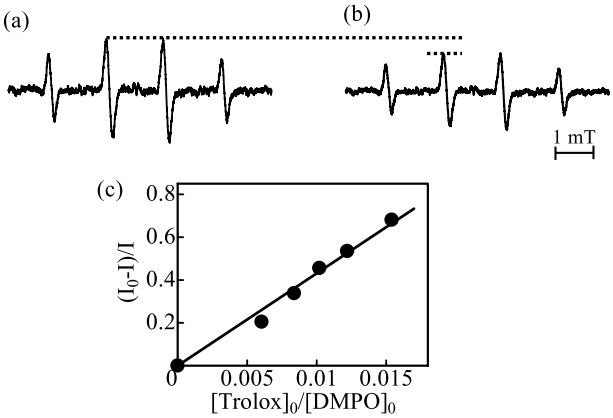

Fig. 3. ESR spectra of RO· radical adduct of DMPO obtained after the UV-photolysis of phosphate buffer containing AAPH (5.0 mM) and DMPO (1.0 mM) in the absence of DM-β-CD: (a) [trolox] = 0, and (b) [trolox] = 8.7×10^{-2} mM. Horizontal broken lines in the spectra demonstrate the change in ESR signal height due to the scavenging activity by trolox. (c) A plot of $(I_0\text{-}I)/I$ against $[Trolox]_0/[DMPO]_0$ in the absence of DM-β-CD using Eq. (1). The slope is equal to trolox's relative scavenging rate constant k_{Trolox}/k_{DMPO}.

(hfsc) was obtained after the analysis with a computer spectrum simulation, resulting in A_N (nitrogen nucleus hfsc) = 1.44 mT and A_H (hydrogen nucleus hfsc) = 1.52 mT.

3.2 Oxygen radical scavenging capacity of trolox

The intensity of the ESR signal was decreased by the presence of trolox, such as shown in Figs. 3a and 3b, indicating that part of the RO· radical was scavenged by trolox. The intensity decrease was quantified and the relative scavenging rate constants were calculated, using Eq. (1). In the absence of DM-β-CD, the ratio of scavenging rate constant (k_{Trolox}/k_{DMPO}) was 43.6 ± 1.4. As the concentration of DM-β-CD was increased, k_{Trolox}/k_{DMPO} monotonically decreased and leveled off when $[DM\text{-}\beta\text{-}CD]_0/[Trolox]_0$ was more than 100 (Fig. 4).

3.3 NMR spectra of trolox/DM-β-CD complex

We utilized 2D ROESY NMR technique to determine the disposition of trolox in the DM-β-CD complex. Figure 5a shows the ROESY NMR spectrum of trolox in D_2O in the presence of DM-β-CD. The proton NMR peaks in trolox were assigned with reference to the NMR study of vitamin E by Baker and Myers [13]. The cross peaks of the H-3,5 protons in DM-β-CD were detected with C(2,7,8)-CH_3 protons as enclosed in the ellipse in Fig. 5. The cross peaks of the inner H-3,5 protons of DM-β-CD with the C(3,4)-H and C(5)-CH_3 protons did not appear, indicating that the moieties of C(2), C(7), and C(8) are encapsulated in the CD cavity, in addition, C(6) in the chromane ring also showed strong interaction with peripheral methyl groups in DM-β-CD. Such observations are consistent

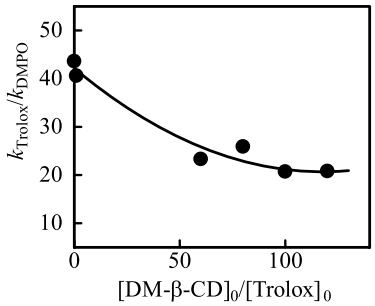

Fig. 4. The relative rate constant (k_{Trolox}/k_{DMPO}) plotted *vs.* [DM-β-CD]$_0$/[Trolox]$_0$: [Trolox]$_0$ = 8.7×10^{-2} mM.

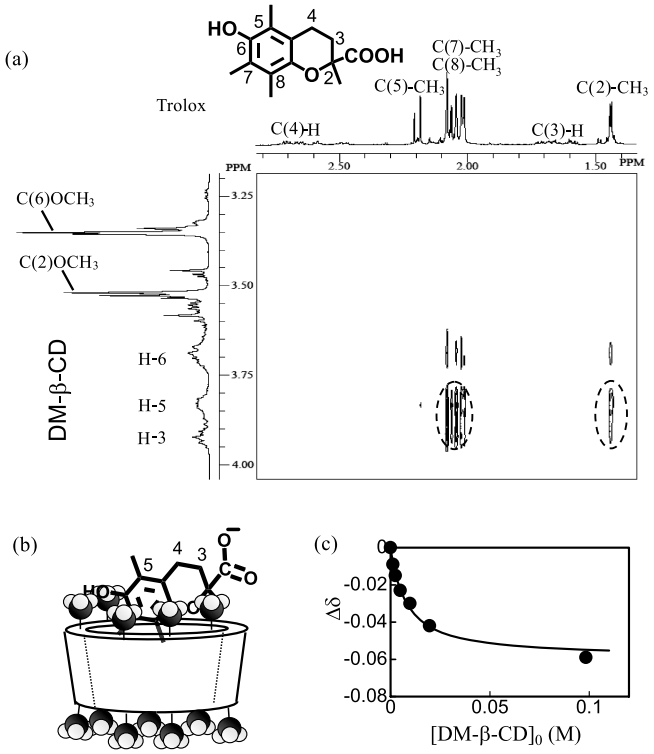

Fig. 5. (a) 2D ROESY NMR spectra (200 ms mixing time) at 303 K in D_2O solution: [trolox] = 1.0 mM and [DM-β-CD] = 10 mM. (b) A plausible structure of the inclusion complex of trolox with DM-β-CD based on NMR results. (c) Changes in chemical shifts of C(2)-CH_3 proton in trolox at various DM-β-CD concentrations.

with the molecular modeling calculation for the trolox/methyl-β-CD inclusion complex [14].

Based on the 2D ROESY NMR results of trolox and DM-β-CD in the complex, the structure of the complex can be speculated. The schematic structure in Fig. 5b illus-

trates that trolox molecule resides in the CD cavity side ways. It should be emphasized that the phenoxyl group in trolox is completely embedded in the entrance portion of CD cavity.

3.4 NMR chemical shift of trolox/CD complexation

Association constant K for trolox : CD inclusion complex can be calculated based on the induced chemical shift in trolox NMR spectrum. Figure 5c shows the chemical shifts for the C(2)-CH_3 protons of trolox as a function of DM-β-CD concentration. The changes in the induced chemical shift leveled off at [DM-β-CD]/[Trolox] larger than 100, indicating that all trolox molecules formed inclusion complex.

The induced chemical shifts $(\Delta\delta)T$ in NMR signals in the 1 : 1 complex formation equilibrium such as:

$$\text{Trolox} + \text{DM-}\beta\text{-CD} \overset{K(\text{trolox})}{\leftrightharpoons} \text{Complex}\,,$$

is given by the following equation [15]:

$$\Delta\delta = \frac{\Delta\delta_{\text{sat}}}{2[\text{Trolox}]_0}\left(\beta - \sqrt{\beta^2 - 4[\text{Trolox}]_0[\text{DM-}\beta\text{-CD}]_0}\right)\,, \tag{2}$$

$$\beta = \frac{1}{K(\text{trolox})} + [\text{Trolox}]_0 + [\text{DM-}\beta\text{-CD}]_0\,, \tag{3}$$

where the $[\ ]_0$ symbol denotes the initial concentration. K(trolox) is an inclusion constant of trolox with DM-β-CD and $\Delta\delta_{\text{sat}}$ is the saturated value of induced chemical shifts. Figure 5c shows the saturation curve for the induced chemical shifts. Using $\Delta\delta_{\text{sat}}$ obtained from Fig. 5c, a nonlinear least-square fit of the induced shifts to Eq. (2) enabled us to determine K(trolox) as $98\pm5\ \text{M}^{-1}$, which is much smaller than those for DM-β-CD complexes of water-insoluble lipophilic antioxidants; for example, $K = 8670$ and $23\,800\ \text{M}^{-1}$ for catechin and epigallocatechin gallate, respectively [16].

4. Discussion

The present experiment demonstrated that upon addition of the solubilizer DM-β-CD, trolox's radical scavenging capacity decreased. In the presence of excess DM-β-CD ($[\text{DM-}\beta\text{-CD}]_0/[\text{Trolox}]_0 = 100$), it decreased by nearly 50% (Fig. 4) and leveled off above this concentration ratio, indicating that all trolox molecules had formed inclusion complexes above this ratio. The objective of this study was to elucidate the cause for this decrease. NMR structure of trolox/DM-β-CD complex revealed that whole trolox molecule was embedded within the wall of methoxy groups at the entrance of the CD cavity. NMR results also indicated that the phenoxyl group in trolox strongly interacts with CD molecule (Fig. 5b), suggesting that trolox is encapsulated in CD so that the phenoxyl group is not exposed to the bulk solution phase. Phenoxyl group is highly reactive to oxygen radical including RO· radical; therefore, the encapsulation of phenoxyl group has made it difficult for RO· radical to contact and react with trolox. This means

that CD in this case is not a mere solubilizer but a reagent that could participate and interfere with the free radical scavenging reaction.

Under *in vivo* conditions, natural proteins and lipids could play a role as solubilizers; therefore, the ORAC values (scavenging rates) of solubilized antioxidants in the laboratory settings are thought to be not too far from *in vivo* situations. However, it may be problematic if trolox is used as a standard in the scavenging assay of CD-solubilized compounds, because trolox ORAC values are CD concentration-dependent. Therefore, one should use trolox ORAC value in the absence of the CD solubilizer as a standard.

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