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Redox Reactions between ABTS•+ and Dihydroxybenzenes as Studied by Cyclic Voltammetry

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Abstract

Redox reactions between several types of polyphenols and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) used in trolox equivalent antioxidant capacity (TEAC) assay were monitored by continuous cyclic voltammetry. TEAC assay is one of the effective methods for clarifying the radical scavenging reaction mechanism of antioxidants. We obtained information on whether the reaction was a simple electron transfer, an electron transfer involving a subsequent chemical reaction of the antioxidant itself, or an electron transfer involving a coupling reaction between $ABTS^{\bullet+}$ and the antioxidant.

Introduction

Polyphenols have a strong reducing power, so they are known to act as antioxidants in the living body. A lot of analytical methods for evaluating the antioxidant activity *in vitro* have been studied.[1] The trolox equivalent antioxidant capacity (TEAC), which shows the scavenging activity against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$), is often used for the antioxidant activity measurement.[1] This method was first developed by Miller *et al*.[2] as a simple and convenient method. Then, it was improved by Re *et al*.[3] and has been widely used to date. Since $ABTS^{\bullet+}$ whose aqueous solution has a green color is decolorized when reduced, the antioxidant capacity can be measured from the decrease in absorbance of the solution.[4] It has been reported that the redox reactions between $ABTS^{\bullet+}$ and antioxidants can be classified into three groups based on the time dependence of the changes in absorbance.[3,5,6] The first group is those involving only a rapid electron transfer, the second is those involving a relatively slow reaction proceeding over several minutes, and the other is a multistep reaction. In addition, catechin, which is a typical polyphenol, has been revealed by LC–MS and NMR to undergo a coupling reaction with $ABTS^{\bullet+}$.[4,7,8] As described above, there are various reaction mechanisms depending on the nature of polyphenols, but the general TEAC analysis does not provide information on such reaction mechanisms.[3] The reaction mechanism is considered important for the correct assessment of antioxidant activity.[9] Occurrence of chemical reaction(s) such as a coupling reaction followed by the electron transfer is one of the important reasons for the poor correlation between TEAC assay results and the number of phenolic hydroxy groups.[4]

In this study, we have traced the electron transfer reaction between $ABTS^{\bullet+}$ and dihydroxybenzenes, which are polyphenolic compounds, by continuous cyclic

voltammetry (CV) measurements. Among the electrochemical techniques, CV is one of the most effective methods for investigating the whole electrode reaction, and it has been reported to provide useful information on redox reactions of antioxidants.[10-13] In this article, it is demonstrated that the redox reactions between ABTS•$^{+}$ and dihydroxybenzenes can be classified in a relatively simple manner *via* CV measurements.

Experimental

Reagents and chemicals

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, potassium persulfate, *p*-hydroquinone (Hyd), resorcinol (Res), chlorogenic acid (Chl), tris(hydroxymethyl)aminomethane (Tris), ethanol, HCl, and KCl were obtained from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Caffeic acid (Caf) was purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-Catechin (Cat) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Other chemicals were of analytical grade and used as received. The chemical structures of these dihydroxybenzenes are shown in Figure S1 in Supporting Information. Ultrapure water for sample preparation was obtained from Millipore Direct-Q UV (Merck, Darmstadt, Germany).

Sample preparation

An ABTS•$^{+}$ aqueous solution was prepared for stock by adding 176 μL of 140 mM potassium persulfate aqueous solution to 30 mL of 2.3 mM ABTS aqueous solution, followed by reacting at room temperature in the dark for 16 h or more.[3] The concentration of ABTS•$^{+}$ was determined using the known molar extinction coefficient {ε = 31100 M^{-1} cm^{-1} in water (pH 7.5) at 414 nm},[4] based on the measurement of the

UV-Vis absorption spectrum with V-650 spectrophotometer (JASCO corp., Tokyo, Japan). A 0.23 mM ABTS•$^{+}$ aqueous solution containing 0.1 M KCl and 40 mM Tris-HCl buffer (pH 7.4) was used for CV measurements. Antioxidants stock solutions were dissolved in water (for Hyd and Res), and ethanol (Caf, Chl, and Cat), respectively.

CV analysis

A glassy carbon (GC) disk electrode (surface area, 0.071 cm^2) (BAS, Tokyo, Japan) was used as the working electrode, an Ag/AgCl (saturated KCl) electrode was used as the reference electrode, and a platinum wire was used as the counter electrode. These electrodes were connected to a potentiostat (ECstat-101, EC Frontier Co., ltd, Kyoto, Japan). CV measurements were performed continuously for about 30 min (900 cycles) at a sweep rate of 1 V s^{-1}. 14.85 mL of ABTS•$^{+}$ sample solution was placed in the measurement vial, and then the voltammetric measurement was started. After several cycles, 0.15 mL of the antioxidant solution (final concentration of 0.1 mM) was added with stirring magnetically. The stirring was stopped 20 seconds after mixing so as not to interfere with the voltammetric measurement.

Results and Discussion

Confirmation of the ABTS and ABTS•$^{+}$ stability during CV measurements

At first, the CV response of ABTS•$^{+}$ was examined. Black lines in Fig. 1 show the CV curve of 0.23 mM ABTS•$^{+}$. The reversible redox response was observed. The reduction peak of ABTS•$^{+}$ to ABTS was observed at 0.45 V and the oxidation peak was observed around 0.55 V. The midpoint potential of 0.50 V was in good agreement with the reported redox potential of ABTS of 0.515 V *vs*. Ag/AgCl.[14] The CV response did not change during the continuous measurement (30 min), suggesting that ABTS and

ABTS•$^{+}$ should be sufficiently stable in the solution tested.

Reaction with Hyd containing a p-dihydroxybenzene moiety

After the addition of Hyd into the ABTS•$^{+}$ solution, oxidation and reduction current peaks due to Hyd redox appeared at 0.35 and −0.1 V, respectively (Figure 1a, red line). The same peak pair was also found in the CV curve obtained with Hyd only (Figure S2a). This redox pair was due to the redox reaction between Hyd and *p*-benzoquinone, and did not change for 30 min after the addition of Hyd (Figure 1a, blue line). The time dependences of the peak current difference, Δi_p, being defined as the difference between the anodic peak current (i_{pa}) and the cathodic peak current (i_{pc}), are shown in Fig. S3 (where Δi_p is expressed as a percentage of the value obtained from the voltammogram of a single substance shown in Fig. S2; The values of i_{pa} and i_{pc} were obtained by subtracting the blank values from the net values). The Δi_p for Hyd remained almost unchanged for 30 min as shown in Fig. S3a (red line). Therefore, it was confirmed that *p*-benzoquinone as the reaction product was stable under the present conditions and the redox reaction was chemically reversible. On the other hand, there was an upward shift in the redox wave of ABTS•$^{+}$/ABTS at 0.45 V for the reduction and 0.55 V for the oxidation (Figure 1a), which suggested an increase in the reduced form (ABTS) produced by the reaction with Hyd. In this case, the peak current difference was constant throughout the measurement (Figure S3a, black line), suggesting that only electron transfer occurred in the reaction between ABTS•$^{+}$ and Hyd, and there was no side reaction occurred.

Reaction with Caf containing an o-dihydroxybenzene moiety

When Caf with an *o*-dihydroxybenzene moiety was added to the ABTS•$^{+}$ solution, the

redox wave appeared at 0.35 V and 0.02 V immediately after the addition (Fig. 1b, red line). This redox wave was the same as that observed in the CV obtained with Caf alone (Fig. S2b), being due to the two-electron transfer of Caf.[13] However, this wave became smaller as the reaction proceeded (Fig. 1b, blue line, and Fig. S3b). This showed that the quinone structure produced by the two-electron oxidation of Caf was involved in the coupling reaction of Caf itself, and was finally electrochemically inactivated.[13] On the other hand, the redox wave of ABTS•$^+$/ABTS at around 0.5 V showed a slight upward shift (Fig. 1b, blue line), and no significant change in the Δi_p value was observed. This suggests that no side reaction occurred in ABTS•$^+$ similar to the result of Hyd (Figure 1a). Thus, it was found that Caf undergoes a coupling reaction by itself through the electron transfer with ABTS•$^+$, but does not occur a coupling reaction with ABTS•$^+$. Chl having an *o*-dihydroxybenzene moiety also showed the same tendency as Caf (Figs. S2e, S3e).

Reaction with Res containing an m-dihydroxybenzene moiety

When Res was added to the ABTS•$^+$ solution, a reduction peak appeared at −0.26 V immediately after the addition (Fig. 1c, red line). This reduction peak current was not observed in the CV curve of Res (Fig. S2c). It was suggested that the oxidation product of Res by ABTS•$^+$ was different from that at the electrode. The reduction wave became smaller with the reaction time (Fig. 1c, blue line, and Fig. S3c), indicating that this oxidation product was unstable, and it produced an electrochemically inactive compound. Furthermore, unlike the above experiments, the oxidation and reduction currents of ABTS•$^+$ also decreased immediately with the addition of Res. It has been reported that ABTS•$^+$ and phloroglucinol having an *m*-dihydroxybenzene moiety formed an adduct.[7] The results shown in Fig. 1c suggested that ABTS•$^+$ and Res also produced

coupling products.

Reaction with Cat containing both m- and o-dihydroxybenzene moieties

When Cat was added to the ABTS•$^{+}$ solution, redox peaks appeared at 0.26 V and 0.09 V immediately after the addition (Fig. 1d, red line). These peaks appeared at almost the same potential in the CV of Cat (Fig. S2d), which were reported to be due to the electron transfer of the B-ring of Cat.[15] Since this current value was much smaller than that expected from the added Cat concentration, it was found that Cat was electrochemically inactivated faster than Caf and Chl (Fig. S3d). On the other hand, the reduction and oxidation currents of ABTS•$^{+}$ decreased slightly immediately after the addition of Cat (Fig. S3d). This tendency is similar to that of Res and can be considered to be due to the reactivity of the *m*-dihydroxybenzene group of the Cat A-ring. This was probably because Cat and ABTS•$^{+}$ undergo a coupling reaction in the A ring.[7] Thus, the reaction between Cat and ABTS•$^{+}$ has been found to exhibit both properties of *o*- and *m*-dihydroxybenzenes. This result shows that Cat undergoes electron transfer reactions with ABTS•$^{+}$ at both A-ring and B-ring.

Conclusions

The redox reactions between ABTS•$^{+}$ and dihydroxybenzenes were observed using CV. CV is a powerful analytical method to study the whole electron transfer reaction including chemical reactions associated with the electron transfer. From the time dependence of CV curves, we can obtain information on whether the redox reaction studied is a simple electron transfer reaction, an electron transfer involving a subsequent chemical reaction(s) of the antioxidant itself, or an electron transfer accompanied with coupling reaction(s) between ABTS•$^{+}$ and antioxidants. It was found that the reaction

mechanism of polyphenols having a dihydroxybenzene group differs depending on the position of the substituent (ortho, meta, or para). In the future, it will be necessary to comprehensively classify the reaction mechanisms of more antioxidants. Simultaneous measurement of absorbance changes and CV curves is also under consideration. In addition, this finding can be applied to understand the reaction of antioxidants with various other oxidants such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•), Fe^{3+}, and reactive oxygen species.

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Supporting Information

This material is available free of charge on the web at http://www.jsac.or.jp/analsci/.

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Figure Caption

Fig. 1 Time changes of the CV curve for the reactions between $ABTS^{\bullet+}$ and antioxidants (a, Hyd; b, Caf; c, Res; d, Cat). Black line, 0.23 mM $ABTS^{\bullet+}$; red line, immediately after adding the antioxidant; blue line, 30 min after adding the antioxidant.

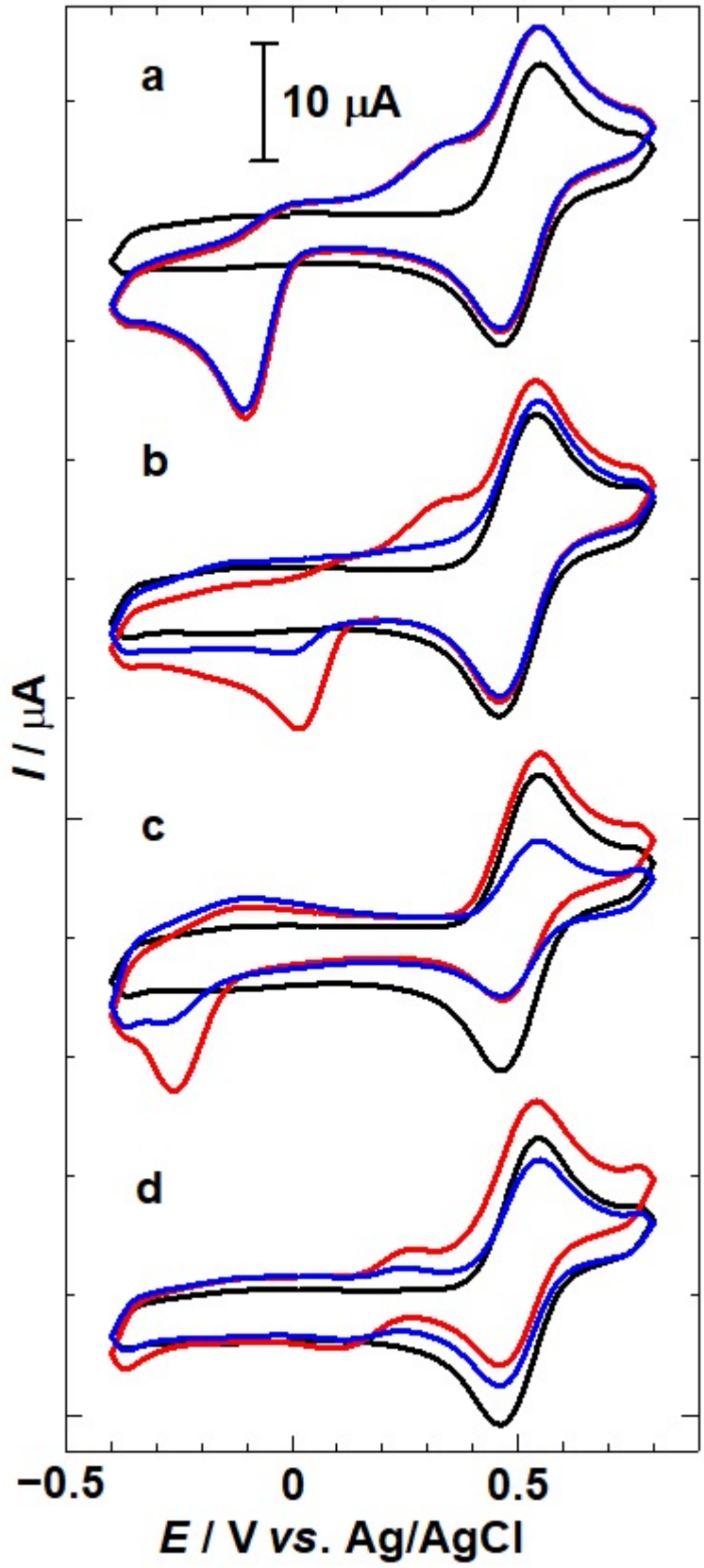

Fig. 1

Graphical Index