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Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation

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Abstract

Radical scavenging activities of 34 natural antioxidants were investigated from an electrochemical viewpoint. While the correlation of the oxidation potentials with their DPPH radical scavenging activities (represented by EC_{50}) was not high (the correlation coefficient, $r = 0.73$), the number of electrons n required for oxidation of an antioxidant, being obtained by continuous flow-column electrolysis with a slower flow rate (0.05 ml min^{-1}), did show a good correlation with EC_{50} ($1/EC_{50} = 1.67n + 0.50$ with $r = 0.94$). The n values of most polyphenols were increased with a decrease in the flow rate, while those of nonpolyphenols were invariant. This suggests that a slower subsequent chemical reaction(s) should be involved in the oxidation of polyphenols, whose higher radical scavenging activities seem to be ascribed to the chemical reactions. In this study, we have proposed a possible mechanism for the oxidation of polyphenols, in which the oxidizable -OH moieties are reproduced through an oxidative dimerization (or more highly polymerization).

Keywords: Polyphenol; Antioxidant; Flow-column electrolysis; Cyclic voltammetry; Radical scavenging activity; Oxidative polymerization

1. Introduction

Polyphenols (e.g., phenolic acids, flavonoids, tannins), being widely distributed in plants, are known to act as antioxidants [1–3]. Also, polyphenols in human diet may exert a beneficial health effect via protecting against some diseases, including coronary heart disease and some cancers [4,5]. Various measurements have been employed to determine the free radical scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazyl) radical [6–13] or active oxygen species [14–16] and the inhibition activity against lipid peroxidation or protein oxidation [17–22] (see review in Ref. [23]), including the in vivo [24,25].

On the other hand, more fundamental approaches with electrochemical measurements have also been employed to evaluate antioxidant capacities of polyphenols [10,18–20,26–33]. Electrochemical measurements may enable us to obtain physicochemical parameters of polyphenols (e.g., redox potential, number of electrons, electron-transfer rate constant, etc.). These parameters seem to possess great potentialities not only for evaluating the antioxidant abilities of polyphenols but also for understanding their reaction mechanisms. Among these parameters, the redox potential, i.e., the reducing power of an antioxidant, could be a key factor that governs its antioxidant activity. Therefore, the oxidation potentials of polyphenols were often measured and then compared to their antioxidant activities including the DPPH radical scavenging activity (the major evaluation method as an initial test) [10] and the inhibition activity of lipid peroxidation, which is more similar to biological systems [10,18–20,26]. So far, some correlations have been reported [10,20,26] for the oxidation potentials of flavonoids (being combined with octanol/water partition coefficients) with the inhibition activity against rat's liver peroxidation [20,26], and also for the oxidation potentials of phenolic acids with the inhibition activity against autoxidation of methyl linoleate or DPPH scavenging activity [10]. Also, the redox properties of

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polyphenols have been utilized as a measure of the antioxidant power of wines on the basis of the measurement of the oxidation current at a constant potential by reversed-phase high-performance liquid chromatography (HPLC) with an electrochemical detector [32]. However, the above correlations between the oxidation potentials of polyphenols and their antioxidant activities were observed only for a certain family of antioxidants having a common structure. It should also be noted that no clear correlations were found between the oxidation potentials of flavonoids and their DPPH scavenging activities or inhibition activities against autoxidation of methyl linoleate [10]. Also, no clear correlation was observed between the oxidation potentials of several natural polyphenols and their inhibition activities of peroxidation of rat synaptosome [18]. Thus, although the oxidation potential would be an important factor for the antioxidant activities of polyphenols, other unclear factor(s) seems to be involved in the activities.

To the best of our knowledge, the ''quantitative'' aspect of electrochemical reactions, i.e., the current magnitude, or the number of electrons (n) involved in the redox reaction has rarely been discussed. In a previous study [34], however, we directed our attention to n values, and then observed ''unusually'' large n values for the oxidation of some polyphenolic antioxidants by means of continuous flow-column electrolysis. Also, we performed digital simulation analyses of cyclic voltammograms for chlorogenic acid (CHL) [34] and caffeic acid (CAF) [35], and then suggested that their electrode oxidations should involve subsequent chemical reaction(s) that is most probably dimerization or even more highly polymerization reactions. Based on these findings, we proposed a possible mechanism for reproduction of oxidizable -OH moieties in polyphenols by their oxidative dimerization (polymerization). It has been claimed that such polymerization reactions should be responsible for their higher antioxidant activities. Consequently, the quantity n seems to be another important factor for determining the antioxidant activities of polyphenols.

In this study, we measured n values for 34 water-soluble naturally occurring antioxidants (flavonoids, phenolic acids, nonphenolics, etc.) by means of flow coulometry using a column electrolytic cell [36]. This method feasibly attains whole electrolysis, in which we can determine n values more accurately than the bulk electrolysis. The coulometric measurements were carried out with various flow rates, so that almost all polyphenols were found to show higher n values at slower flow rates. The correlation study of the n values at a slow flow rate with the DPPH radical scavenging activity has suggested that the radical scavenging activity is enhanced by comparatively slow chemical reaction(s), i.e., dimerization, which follows the oxidation of polyphenols. Based on these findings, a new electrochemical method is being proposed for the evaluation of radical scavenging activity of antioxidants.

2. Materials and methods

2.1. Materials

The antioxidants tested in this study are listed in Fig. 1. Catechol, *p*-hydroquinone, pyrogallol, ellagic acid, and ascorbic acid were purchased from Wako Pure Chemical Industries, Ltd.; rosmarinic acid, (+)-taxifolin, luteolin, and eriodictyol were from Funakoshi Co., Ltd.; (−)-epicatechin, apigenin, chrysin, and biochanin A were from Aldrich; protocatechuic acid and cysteine were from Nacalai Tesque, Inc.; curcumin was obtained from Biolink Co.; all other antioxidants and DPPH radical were purchased from Tokyo Kasei Kogyo Co., Ltd. All these compounds were used as received. Other reagents were of the highest grade available.

2.2. Evaluation of radical scavenging activities of antioxidants by DPPH radical

Radical scavenging activities of antioxidants were determined using DPPH radical [7]. Aliquots of an 1.0 mM antioxidant solution (for ellagic acid (**31**), a 0.2 mM solution was used because of its low solubility) in 1:1 (v/v) water–ethanol containing 50 mM KCl and 50 mM phosphate buffer (pH 7.0; this medium was also used for electrochemical measurements) were added to a 0.1 mM DPPH solution in the same buffer; the concentration of the antioxidant was changed in the range from 0 (control) to 0.1 mM (7 to 15 samples were used for one antioxidant). The reaction mixtures were incubated in capped bottles in a water bath at 25 °C for 5 h while stirring with a magnetic stirrer (H+P Labortechnik GmbH, HP46007). After this reaction time, radical scavenging reactions for almost all antioxidants reached to their steady states. Then, the decrease in the absorbance of DPPH at 525 nm (absorption maximum) with the addition of antioxidants was monitored by a UV–Vis spectrophotometer (Hitachi U-3210). The DPPH concentration was obtained from the absorbance by using the molar absorption coefficient ($\varepsilon_{max} = 12\,000$). The radical scavenging activity (EC_{50}) was then evaluated by using a conventional method [7], which is defined by the ratio of the antioxidant concentration necessary for decreasing the initial DPPH concentration by 50% to the initial DPPH concentration (the actual decrease in absorption induced by the test compound was calculated by subtracting that of control). Note that a smaller value of EC_{50} means a higher antioxidant activity. In addition to EC_{50}, the average stoichiometric number (n_{DPPH}) of DPPH in the reaction with each antioxidant was evaluated from the spectrophotometric data with different antioxidant concentrations.

2.3. Electrochemical measurements

Cyclic voltammetry and flow-column electrolysis were performed by a conventional three-electrode system using

No.	Compounds	Substituent	Stuructures
	benzenes		
1	catechol	1,2–OH	
2	resorcinol	1,3–OH	
3	*p*-hydroquinone	1,4–OH	
4	pyrogallol	1,2,3–OH	
	benzoic acids		
5	*p*-hydroxybenzoic acid	4–OH	
6	vanillic acid	3–OCH_3, 4–OH	
7	syringic acid	3,5–OCH_3, 4–OH	
8	protocatechuic acid	3,4–OH	
9	gentisic acid	2,5–OH	
10	gallic acid	3,4,5–OH	
	cinnamic acids		
11	*p*-coumaric acid	4–OH, R = H	
12	ferulic acid	3–OCH_3, 4–OH, R = H	
13	sinapinic acid	3,5–OCH_3, 4–OH, R = H	
14	caffeic acid	3,4–OH, R = H	
15	chlorogenic acid	3,4–OH, R = quinic acid	**16**
16	rosmarinic acid		
	Flavans		
17	(+)-catechin	3,5,7,3',4'–OH	
18	(−)-epicatechin	3,5,7,3',4'–OH	
	Flavonols		
19	quercetin	3,5,7,3',4'–OH	
20	morin	3,5,7,2',4'–OH	
21	rutin	5,7,3',4'–OH, 3–rutinose	
22	kaempferol	3,5,7,4'–OH	
	Flavones		
23	chrysin	5,7–OH	
24	apigenin	5,7,4'–OH	
25	luteolin	5,7,3',4'–OH	
	Flavanones		
26	naringenin	5,7,4'–OH	
27	hesperetin	5,7,3'–OH, 4'–OCH_3	
28	eriodictyol	5,7,3',4'–OH	
29	(+)-taxifolin	3,5,7,3',4'–OH	
	Isoflavanone		
30	biochanin A		**30** **31**
	tannin		
31	ellagic acid		
	others		
32	curcumin		**32**
	non-polyphenols		
33	ascorbic acid		**33** **34**
34	cysteine		

Fig. 1. Structures of antioxidants.

a laboratory-constructed microcomputer-controlled system, in which a potentiostat (Hokuto Denko, HA1010mM1A) was used for controlling the working electrode potential.

For cyclic voltammetry, a plastic formed carbon (PFC) electrode of a surface area = 0.071 cm^2, an Ag/AgCl (saturated KCl) electrode, and a platinum coil electrode were used as the working, reference, and counter electrodes, respectively. The electrochemical cell was thermostatted at 25 ± 0.1 °C. Before each voltammetric measurement, the PFC electrode was fleshly polished with a lapping film (Maruto, 0.3 μm), followed by rinsing with distilled water in an ultrasonic field. Test solutions were purged with N_2 gas prior to the voltammetric measurements.

The continuous-flow-column electrolysis was done using a commercially available column electrolytic cell (Hokuto

Table 1
DPPH scavenging activities and electrochemical parameters of antioxidants

No.	Compounds	DPPH method		Cyclic voltammetry		Flow-column electrolysis
		EC_{50}	n_{DPPH}	E_{pa} [a]	I_{pa} [b]	n [c]
1	catechol	0.22	2.3	0.240	13.4	2.1
2	resorcinol	0.93	0.63	0.632	11.0	0.04
3	*p*-hydroquinone	0.37	1.4	0.207	7.5	1.9
4	pyrogallol	0.10	4.9	0.160	18.0	5.5
5	*p*-hydroxybenzoic acid	>10	0	0.747	12.0	0
6	vanillic acid	1.0	0.48	0.524	10.0	0.01
7	syringic acid	0.42	1.2	0.336	9.7	2.1
8	procatechuic acid	0.22	2.2	0.318	6.8	1.6
9	gentisic acid	0.29	1.7	0.221	12.0	1.9
10	gallic acid	0.15	3.1	0.233	14.2	4.6
11	*p*-coumaric acid	1.6	0.38	0.583	7.5	0.04
12	ferulic acid	0.44	1.2	0.430	6.8	1.3
13	sinapinic acid	0.30	1.6	0.314	6.9	2.0
14	caffeic acid	0.20	2.4	0.212	9.0	2.2
15	chlorogenic acid	0.14	3.6	0.261	9.0	2.1
16	rosmarinic acid	0.12	3.5	0.266	14.7	4.7
17	(+)-catechin	0.13	3.8	0.215	9.8	3.2
18	(−)-epicatechin	0.12	4.3	0.218	11.0	3.6
19	quercetin	0.14	3.7	0.178	10.0	4.5
20	morin	0.25	2.1	0.203	7.0	1.8
21	rutin	0.18	2.9	0.360	8.0	3.8
22	kaempferol	0.50	1.1	0.242	7.6	1.6
23	chrysin	>10	0	0.794	5.8	0
24	apigenin	>10	0	0.658	8.6	0
25	luteolin	0.20	2.5	0.306	11.0	3.4
26	naringenin	>10	0	0.688	9.0	0
27	hesperetin	1.19	0.45	0.524	5.1	0.04
28	eriodictyol	0.16	3.1	0.240	11.0	3.3
29	(+)-taxifolin	0.16	3.1	0.248	9.4	2.8
30	biochanin A	>10	0	0.912	6.4	0
31	ellagic acid	0.10	5.0	0.361	30.5[d]	5.8
32	curcumin	0.24	2.1	0.390	4.8	2.6
33	ascorbic acid	0.25	2.0	0.167	7.4	2.0
34	cysteine	0.51	0.96	0.530	4.2	0.84

[a] The anodic peak potential in V vs. Ag/AgCl.
[b] The anodic peak current for 1.0 mM in μA.
[c] The n values were measured at a slower flow rate (0.05 ml s^{-1}).
[d] Estimated from the I_{pa} value obtained at 0.2 mM.

Denko Co., Ltd., HX-201), which consists of a carbon fiber working electrode tightly packed in a porous Vycor® glass tube (efficient internal volume; 2 cm^3, surface area; approximately 2000 cm^2), an Ag/AgCl (saturated KCl) reference electrode, and a platinum coil counter electrode. An HPLC pump (Jasco 880-PU) was used to deliver a carrier solution to the column electrolytic cell. For determination of the number of electrons, an aliquot (20 μl) of a sample solution containing 1.0 mM (or 0.2 mM for **31**) antioxidant was introduced via a sample injector into the column cell. During the measurement, a constant potential was applied to the carbon fiber working electrode. The mean n value was obtained from three repeated measurements. The flow electrolysis was carried out at room temperature (25 ± 5 °C). We used potassium ferrocyanide as a standard to confirm perfect electrolysis.

3. Results

Table 1 shows the values of EC_{50} and n_{DPPH} for the antioxidants examined. Cyclic voltammograms of some polyphenolic antioxidants are shown in Fig. 2. It is obvious that there are marked differences in the oxidation potential among the polyphenols having different structures. Several antioxidants with two oxidizable moieties (e.g., **17**, **31**) had two oxidation peaks. However, since the first oxidation wave rather than the second one is primarily responsible for the radical scavenging activity, we have tentatively directed our attention to the first oxidation wave. The anodic peak potential (E_{pa}) and current (I_{pa}) for the first wave at 20 mV s^{-1} of each antioxidant are shown in Table 1. Then, the correlation of these electrochemical parameters (E_{pa} and I_{pa}) with EC_{50} was investigated by a linear regression analysis. In the analysis, we employed the reciprocal of EC_{50}, which represents the radical scavenging activity. Regarding the anodic peak potential, its exponential value was tentatively employed, because it should be related to the concentration of the redox species by the Nernst equation:

$$\frac{C_{Ox}}{C_{Red}} = \exp\left[\frac{nF}{RT}(E - E^{\circ\prime})\right] \quad (1)$$

where C_{Ox} and C_{Red} are the concentrations of the oxidized and reduced forms, respectively, E is the electrode potential, and $E^{\circ\prime}$ is the formal potential of the redox couple.

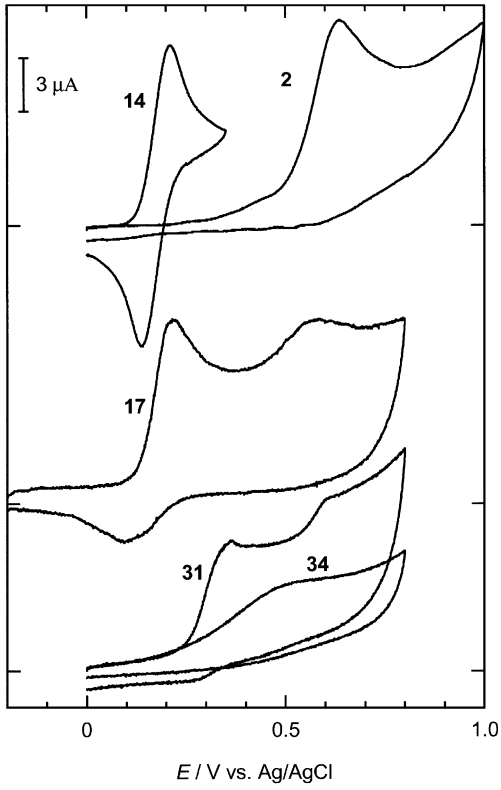

Fig. 2. Cyclic voltammograms of 1.0 mM (0.2 mM for **31**) antioxidants at the PFC electrode in 1:1 (v/v) water–ethanol containing 50 mM KCl and 50 mM phosphate buffer (pH 7.0). The scan rate: 20 mV s^{-1}.

Fig. 3a shows the result of the regression analysis between $1/EC_{50}$ and exp E_{pa}. As seen in the figure, however, only a low correlation with the correlation coefficient (r) = 0.73 was obtained. Although the unaltered value of E_{pa} was also used instead of its exponential value, only an equivalent correlation (r = 0.74) was obtained. Previous authors [19,20,26] employed another potential parameter, i.e., the half peak potential ($E_{pa/2}$) of the first oxidation wave of the cyclic voltammogram [19], or the half-wave potential ($E_{1/2}$) of the first oxidation wave determined by using flow coulometry [20,26]. We also examined the correlations of $1/EC_{50}$ with $E_{1/2}$ and $E_{pa/2}$; however, their correlations, though being examined only for phenolic acids (14 samples), were also not high (i.e., r = 0.82 and 0.81, respectively, for $E_{1/2}$ and $E_{pa/2}$).

Furthermore, the first oxidation peak current, I_{pa}, was used as an additional variable to the analysis with E_{pa}, and a multiple regression analysis was performed. Slight improvement of the correlation was achieved as shown in Fig. 3b, the regression equation being $1/EC_{50} = -5.60 \exp E_{pa} + 0.294\ I_{pa} + 9.47$ with r = 0.86.

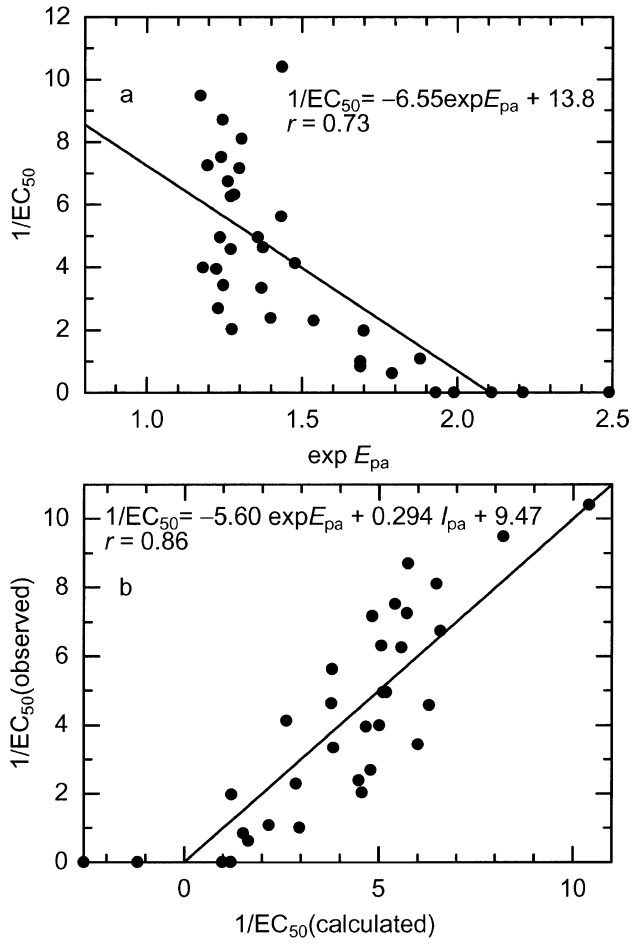

Fig. 3. Correlations of the electrochemical parameters of antioxidants with their DPPH scavenging activities. (a) Plots of $1/EC_{50}$ against exp E_{pa}; (b) plots of the observed values of $1/EC_{50}$ against the calculated values using the regression equation employing exp E_{pa} and I_{pa} as independent variables.

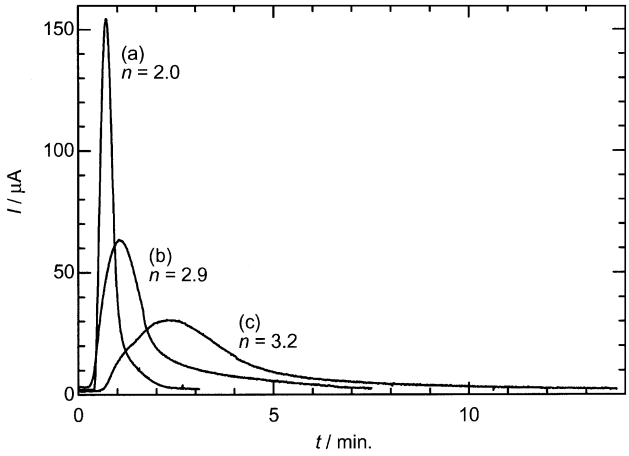

Fig. 4. Current–time curves in the flow-column electrolysis of (+)-catechin (**17**) at 340 mV (vs. Ag/AgCl) at different flow rates: (a) 1.0 ml min^{-1}; (b) 0.2 ml min^{-1}; (c) 0.05 ml min^{-1}. Number of electrons (n) can be calculated from the peak areas.

Fig. 4 shows the current–time curves in the flow-column electrolysis of (+)-catechin (**17**) at three different flow rates (0.05, 0.2, and 1.0 ml min^{-1}). The applied potential was set at 340 mV vs. Ag/AgCl, which corresponds to the $E_{1/2}$ ($\approx E_{mid}$) of DPPH radical (see Discussion). As seen in Fig. 4, the peak area became larger with a decrease in the flow rate, showing an increase in the n values (from 2.0 to 3.2) for the oxidation of **17**. The n values were obtained from the

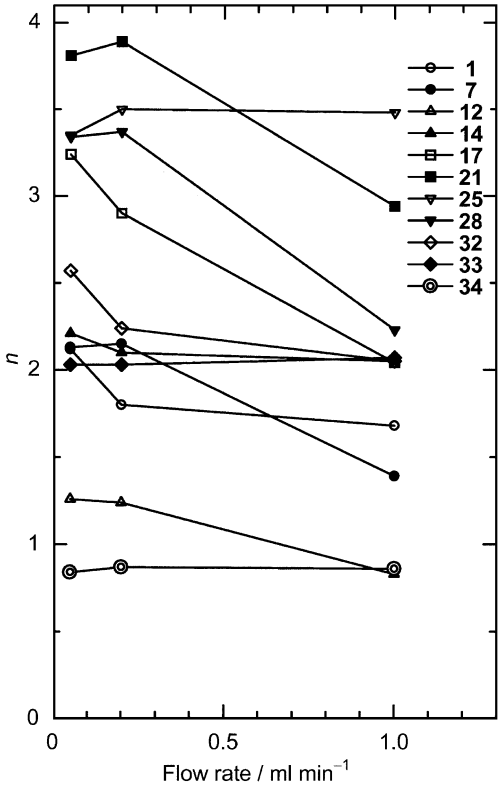

Fig. 5. Flow-rate dependences of n in flow-column electrolysis for various antioxidants. The applied potential: 340 mV vs. Ag/AgCl.

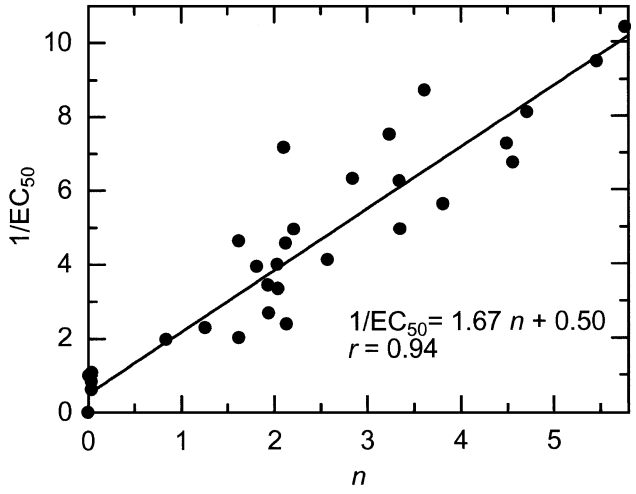

Fig. 6. Correlation of the DPPH scavenging activity (EC_{50}) of polyphenols with their n values determined by flow-column electrolysis with a slower flow rate (0.05 ml min^{-1}). The applied potential: 340 mV vs. Ag/AgCl.

current–time curves corrected for the base current by using Faraday's law. Such a flow-rate dependence of n suggested that a subsequent chemical reaction(s) following the electrochemical oxidation should contribute to the increase in n, and that the resulting compound(s) should be further oxidized.

While the n values of nonpolyphenols (**33** and **34**) were not changed with the flow rate, most polyphenols (except a few ones, e.g., **15**, **25**, and **31**) showed certain increase in n with lowering the flow rate in the same manner as **17**. Some examples are shown in Fig. 5. As seen in the figure, the degree of the increase in n differed from one antioxidant to another, but it was suggested that, for most polyphenols, subsequent chemical reactions should be involved in the increase in n. We assumed that the n values obtained at the slowest flow rate (0.05 ml min^{-1}) should give important "quantitative" information about the antioxidant activity, and then carried out a regression analysis by using EC_{50} and the n values at 0.05 ml min^{-1}. It should be stressed that an excellent correlation was successfully obtained with these two parameters ($r=0.94$), as shown in Fig. 6. And also, the n_{DPPH} values determined by the DPPH method were generally very close to the n values, as shown in Table 1 ($n_{DPPH}=0.809n+0.290$, $r=0.93$).

4. Discussion

4.1. On evaluation of the radical scavenging capacity of antioxidants by cyclic voltammetry

The oxidation potential of polyphenols has been regarded as an important factor for their antioxidant activities; therefore, it has been often used for the evaluation of the antioxidant activity [10,18–20,26,27,29–32]. Cyclic voltammetry is one of the most useful methods of evaluating the oxidation potential. Accordingly, in this study, we investigated the contributions of some potential parameters including E_{pa} and $E_{pa/2}$ and also $E_{1/2}$ obtained from flow coulometry. However, each potential parameter showed a lower correlation coefficient than that observed in Fig. 6.

Thus, it was suggested that the oxidation potential of polyphenols, i.e., how easy they are oxidized, was certainly an important factor for determining their radical scavenging activities, but it should not be "omnipotent". We included another parameter, I_{pa}, in the regression analysis, since I_{pa} would have "quantitative" information about the electrode reaction. In practice, slight, but certain improvement of the correlation was obtained. Although I_{pa} itself showed no clear correlation with EC_{50} ($r=0.55$), this factor seems to play an important role in determining EC_{50} on the quantitative side.

Nevertheless, the electrochemical parameters obtained by cyclic voltammetry could show no better correlation with EC_{50} than those obtained by flow-column electrolysis. This is probably because the time scale of the voltammetric measurements was relatively short. In the cyclic voltammetric measurements, it took only about 1 min for one scan (at 20 mV s^{-1}). This measurement time is very short compared with the total measurement time in the flow-column electrolysis. It seems that the effect of the possibly slow chemical reactions following the electrochemical oxidation was not reflected in the cyclic voltammograms.

4.2. Effect of chemical reactions for the DPPH radical scavenging reaction

In view of obtaining electrochemical parameters reflecting the effect of the chemical reactions, we performed flow coulometric measurements with slower flow rates. Then, the n values of the antioxidants were evaluated through the constant potential electrolysis at 340 mV vs. Ag/AgCl. This potential corresponds to the midpoint potential (E_{mid}) between the anodic and cathodic peak potentials of the cyclic voltammogram of DPPH radical, which could be approximated to the formal potential, $E^{\circ\prime}$. At this potential, DPPH radical should be oxidized by 50% at equilibrium in the absence of antioxidants (see Eq. (1)).

As expected, the n values determined at 340 mV with a slower flow rate (i.e., 0.05 ml min^{-1}) showed a very good correlation with the EC_{50} values for DPPH radical scavenging reaction (Fig. 6). This suggests that the n values determined should involve a considerable contribution from the chemical reactions following the electrochemical oxidation of the antioxidants, and hence were intimately related to their DPPH radical scavenging activities.

What happened during the flow electrolysis with the slower flow rate? To answer this question, we have examined the effects of the applied potential and the flow rate. In Fig. 7, the coulomb (i.e., n)-potential curves obtained at a relatively high flow rate (1.0 ml min^{-1}) are shown for some phenolic acids as well as DPPH. In principle, the coulomb-

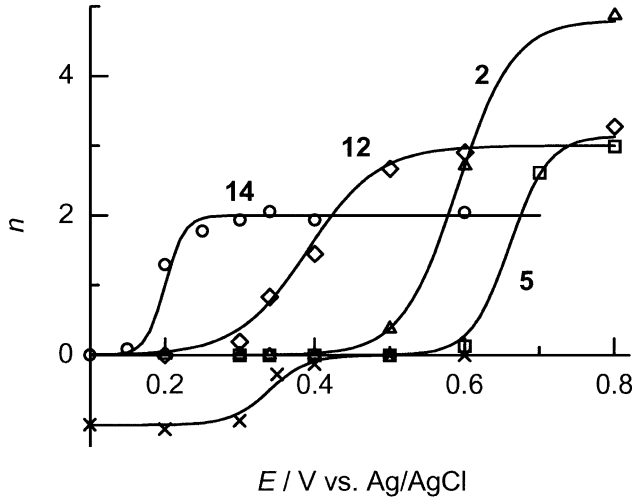

Fig. 7. Coulomb (n)-potential curves of some antioxidants and DPPH. The n values were determined by flow-column electrolysis at a higher flow rate (1.0 ml min^{-1}). △, resorcinol (**2**); □, *p*-hydroxybenzoic acid (**5**);◇, ferulic acid (**12**); ○, caffeic acid (**14**); ×, DPPH radical.

potential curves of a pair of reduced and oxidized species (here, an antioxidant and DPPH) must overlap each other, or give positive and negative values of n in a definite potential range for the sake of electron exchange between the two species. As seen in Fig. 7, ferulic acid (**12**) and CAF (**14**) showed a distinct overlap in their coulomb-potential curves; this is consistent with their reactivities with DPPH (the EC_{50} values of **12** and **14** being 0.44 and 0.20, respectively). On the contrary, the coulomb-potential curve of hydroxybenzoic acid (**5**) showed no overlap with that of DPPH, and hence **5** could not react with DPPH (EC_{50}>10). However, while resorcinol (**2**) could react with DPPH (EC_{50}=0.93), its curve showed little overlap with that of DPPH in the diagram shown in Fig. 7. This may be explained in the following manner.

In the previous study [34], we reported that the n values of CHL and CAF were increased by chemical reactions that followed the electrochemical oxidations. In flow-column electrolysis, the effect of the subsequent chemical reaction is generally enhanced by an increase of electrolysis time, i.e., by slowing down the flow rate. In Fig. 8, the variation of the coulomb-potential curve with the flow rate is shown for **2**, **5**, and **14**. As seen in the figure, the coulomb-potential curves shifted to more negative potentials, or showed larger n values at a constant potential with a decrease in the flow rate, while the coulomb-potential curve of DPPH was not influenced by the flow rate. Thus, at the slower flow rate (i.e., 0.05 ml min^{-1}), the oxidation current for **2**, though rather small, was observed even at the lower electrolysis potential of 340 mV ($=E^{\circ\prime}$ for DPPH radical); see the inset in Fig. 8. Such a flow-rate dependence of the coulomb-potential curve may be explained by assuming a slow subsequent chemical reaction(s). Also, it is suggested that the product(s) of this chemical reaction(s) should be furthermore oxidized. In this manner, the reactivity of **2** with DPPH seems to be understood by considering the shift of the coulomb-potential curve with a decrease in the flow rate. We would like to add that the coulomb-potential curve of the antioxidant having a higher oxidation potential (e.g., **5**) had no overlap with that of DPPH even at the slowest flow rate (see also Fig. 8); this is in harmony with the lower reactivity of **5** with DPPH radical (with the EC_{50} value of >10 as shown in Table 1).

4.3. On the chemical reactions

It is generally known that polyphenols undergo dimerization reaction(s) upon their oxidation [29,37–42]. The oxidized products of CAF (**14**) in the autoxidation [37] and the chemical oxidation [38] have been reported. On the other hand, we have reported that the seeming two-electron oxidation of **14** occurs stepwise via one-electron processes, each of which follows an irreversible chemical reaction(s) [35]. From these findings, we have proposed possible oxidation mechanisms for **14** in the previous papers [34,35]. In the proposed mechanisms, the semiquinone radical (and possibly the one-electron oxidation product) undergoes a dimerization reaction. By using the reaction mechanism proposed in an early report [35], however, the observed n value larger than the number (=2) of -OH moieties in **14** cannot be explained. We showed previously [34] an alternative reaction mechanism that can explain the larger n value. It is generally recognized that various oxidation products are produced through oxidation of a polyphenol. Especially for the electrochemical oxidation, the coupling reaction seems to proceed intensively so that a variety of oxidation products including dimers might be produced in the electrolyzed solutions. In this study, another possible reaction mechanism involving a different oxidized

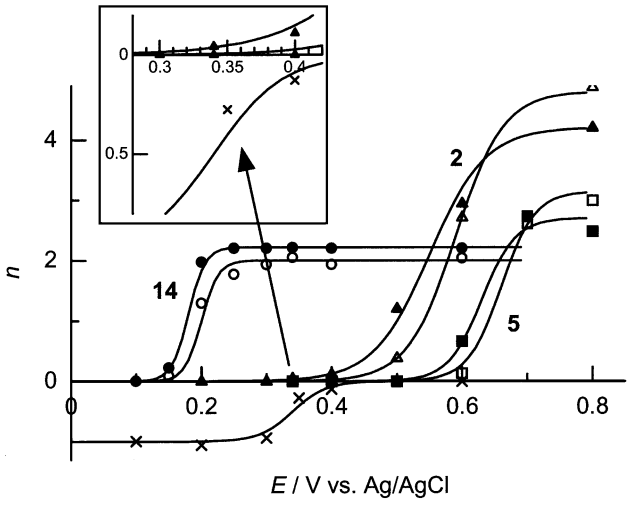

Fig. 8. Flow-rate dependences of the coulomb (n)-potential curves for resorcinol (**2**), *p*-hydroxybenzoic acid (**5**), and caffeic acid (**14**). Flow rate: (△, □, ○) 1.0 ml min^{-1}; (▲, ■, ●) 0.05 ml min^{-1}. DPPH radical (×) showed no flow-rate dependence. The inset shows a magnified figure of the curves around 0.34 V.

product reported in a literature [38] is presented in Fig. 9. At first, the semiquinone radical (having resonance structures) is produced by one electron oxidation. A coupling product [38] of the two semiquinone radicals is shown by **A**. It should be noted that a water molecule participates in the dimerization reaction, so that two -OH moieties are reproduced. Although dimer **A** is a two-electron oxidation product, it possesses still four -OH moieties. Therefore, **A** may be further oxidized to **B** by four electrons. After all, three electrons are involved in the oxidation of one molecule of parent **14** to **B**. On the other hand, another reaction mechanism in which *o*-quinones are dimerized was reported [40–42]. Such a reproduction mechanism of -OH moieties seems to be applicable to many other polyphenolic antioxidants. The larger *n* values of polyphenols are most probably due to the reproduction of -OH moieties by chemical reactions. Since the chemical reactions are rather slow compared with the electrochemical oxidation processes, most polyphenols examined seem to have shown the flow-rate dependence of *n* as in Fig. 5. Although a few exceptional polyphenols (e.g., **15**, **25**, and **31**) had constant *n* values against the flow rate, it is conceivable that the chemical reactions for **25** and **31** are too rapid to show the flow-rate dependence of *n*, and that the chemical reaction for **15** is not observed owing to the relatively low pH (=7.0) of the medium; the increase of *n* probably due to chemical reactions was observed at higher pH (>8) [34]. As regards the nonpolyphenols **33** and **34**, it is well known that the antioxidants undergo subsequent chemical reactions in their oxidation (i.e., decomposition for **33** [43,44] and S–S coupling for **34** [45]). Because these chemical reactions are very rapid, the oxidation products are rapidly inactivated against further oxidation.

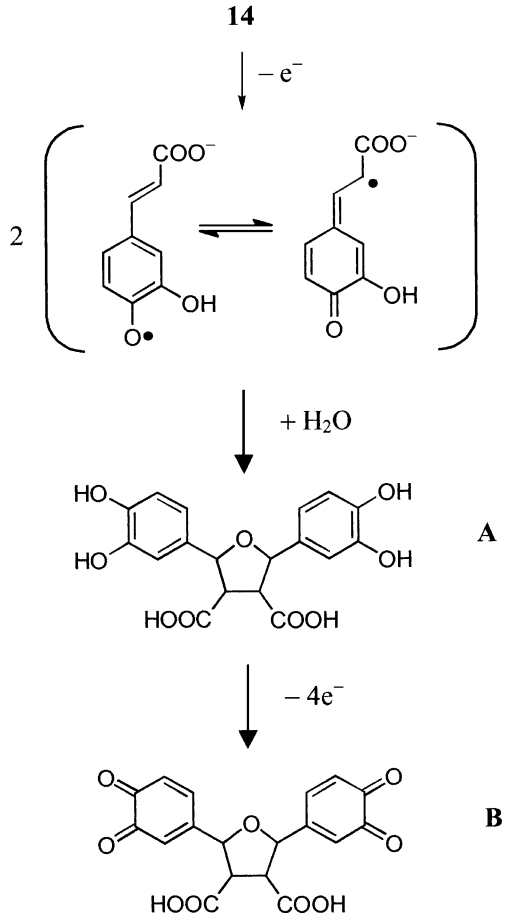

Fig. 9. A possible mechanism of the oxidative dimerization of a representative polyphenol (**14**).

4.4. A new approach to the evaluation of radical scavenging activity of antioxidants

In this study, it has been found that the EC_{50} and n_{DPPH} values from the DPPH method show good correlations with the *n* values obtained by the column electrolysis with a slower flow rate. Thus, either the *n* values or the EC_{50} values, being evaluated with longer reaction times, seems to reflect the radical scavenging activities of antioxidants in an "adequate" manner, i.e., by including the contributions from subsequent chemical reactions following the electrochemical oxidation. Accordingly, the flow-column electrolysis can be used as a substitute for the DPPH method that has so far been extensively employed as a facile method for the evaluation of radical scavenging activities of antioxidants. Although the column electrolysis has been already employed for this purpose [10,20,26,28], we would like to stress that the electrolysis should be carried out with sufficiently slow flow rates. Notwithstanding, it takes less than 10 min to process one sample in the present flow system, and only a small amount of sample (20 μl aliquots of a 1.0 mM antioxidant solution) is required. Also, it should be noted that the flow system would be useful for spectrophotometric or electrochemical characterization of oxidation products [34]. The present flow system looks promising in the development of a new evaluation system for radical scavenging activities of antioxidants.

Finally, we would like to address the correlation of the *n* values with the inhibition activity against lipid peroxidation. In this study, we have measured the *n* values at 340 mV ($=E^{\circ\prime}$ for DPPH radical) in order to compare them to the DPPH radical scavenging activities. Nevertheless, the *n* values determined showed some correlations with the inhibition activity against autoxidation of methyl linoleate (e.g., $r=0.76$ [46] or 0.71 [10]). In many cases [19,20,47,48], however, they showed low correlations. This is probably because the inhibition activity against lipid peroxidation is usually evaluated by using micelle systems [23], in which electron exchange seems to occur at the micelle interface. This inference is supported by the previous study [20] in which the octanol/water partition coefficient as well as the oxidation potential has been found to be an important parameter in determining the inhibition activities of flavonoids against rat's liver peroxidation. For understanding such heterogeneous electron-transfer pro-

cesses, the polarizable oil/water interface [49] is very useful as the simplest model of biomembrane. Recently, the electron transfer of ascorbic acid at the polarizable oil/water interface has been studied by means of voltammetric techniques [50–52]. Such a new approach would lead to a better understanding of the radical scavenging activities of antioxidants in vivo.

5. Conclusion

(1) The oxidation potentials measured for 34 natural antioxidants by cyclic voltammetry have shown a lower correlation ($r=0.73$) with the DPPH radical scavenging activities. However, a certain improvement ($r=0.86$) has been achieved by introducing the anodic peak potential (I_{pa}) as an additional variable, showing importance of the quantitative factor in the DPPH scavenging activities.

(2) The number of electrons (n) required for oxidation of a polyphenolic antioxidant, being measured by flow-column electrolysis, has generally been found to increase with the electrolysis time. This suggests that some chemical reaction(s) (e.g., dimerization) following the oxidations of a polyphenol regenerates the oxidizable -OH moieties in the oxidation product. The n values being determined at a lower flow rate shows a higher correlation ($r=0.94$) with their DPPH scavenging activities. Thus, subsequent chemical reaction(s) most probably enhance the antioxidant activities of the polyphenols.

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