A correlation between the superoxide anion scavenging capacity of antioxidants and their antioxidant capacity as measured by the galvinoxyl or DPPH method

(Received October 26, 2012)
(Accepted January 21, 2013)

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Abstract

The superoxide anion scavenging capacity of 11 antioxidants (AH) was observed, and the results were compared with the antioxidant capacity as measured by DPPH and galvinoxyl methods. The superoxide anion was generated by the xanthine/xanthine oxidase system. 4-[3-(4-iodophenyl)-2-(4- nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate sodium salt (WST-1) was used as a probe of the superoxide anion. The color development rates of WST-1 were measured under various [AH]/[WST-1] ratios, and the data obtained were analyzed according to the following formula.

\[ R = \frac{\Delta A_0 - \Delta A}{\Delta A} = 1 + (k_A/k_W)([AH]/[WST-1]) \]

where \( \Delta A_0 \) and \( \Delta A \) are the color development rates of WST-1 in the absence and presence of antioxidants, respectively, and \( k_A \) and \( k_W \) are the rate constants of the reaction of superoxide anion with the antioxidants and WST-1.

The slope \( k_A/k_W \) calculated from the linear regression of the plot of \([AH]/[WST-1]\) vs. \( V_0/V \), indicated the relative superoxide anion scavenging capacity of each antioxidant.

The superoxide anion scavenging capacity decreased in the order of caffeic acid, n-propyl gallate, 7,8-dihydroxyflavone, gallic acid, catechin, pyrogallol, quercetin, L-ascorbic acid, BHA, 4-hydroxycoumarin and ferulic acid. Correlation coefficients between the superoxide anion scavenging capacity and the antioxidant capacity as measured by the galvinoxyl or DPPH method were as low as 0.448 (galvinoxyl method) and 0.368 (DPPH method), and it was shown that the antioxidant capacity measured by DPPH method or galvinoxyl method does not necessarily reflect the scavenging capacity of superoxide anion.

Keywords: antioxidant capacity, superoxide anion, DPPH, galvinoxyl, WST-1

I Introduction

Over the past few decades, many researchers have focused on natural antioxidants in food not only because of the impact of oxidation on the flavor of food, but also because it had been widely recognized that antioxidants are important health-protecting factors. However, screening antioxidants in food, which involves the separation of each antioxidant compound and studying it individually, is costly and inefficient. Therefore, a convenient method for the quick quantitation of antioxidant effectiveness in preventing diseases is appealing for researchers, and many methods to measure antioxidant capacity have been reported. Depending upon the reactions involved, these assays can roughly be classified into two types: assays based on hydrogen atom transfer (HT) reactions and assays based on electron transfer (ET) reactions.

Many hydrogen atom transfer based assays have been reported and include the inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC)\(^1\), total radical trapping antioxidant parameter (TRAP)\(^2\), and crocin bleaching assays\(^3\).

Electron transfer reaction based assays include the total phenols assay using Folin–Ciocalteu reagent\(^4\), Trolox equivalence antioxidant capacity (TEAC)\(^5\), and ferric ion reducing antioxidant power (FRAP)\(^6\).

Assays using stable radicals such as \( \alpha,\alpha\)-diphenyl-\( \beta\)-picrylhydrazyl (DPPH)\(^7\), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)\(^8\), or N,N-dimethyl-p-phenylene- diamine...
dihydrochloride radical cation (DMPD) have also been widely employed. In our previous study, we proposed a new procedure for an assay using galvinoxyl radicals.

These assays are believed to involve both HT and ET mechanisms; therefore, they are convenient especially for analyzing the total antioxidant capacity of foods, which may contain various types of antioxidants. Moreover, they are technically simple and can be carried out using a non-expensive spectrophotometer.

However, some disadvantages limit its applications. Stable radicals are usually long-lived nitrogen or oxygen radicals that bear little similarity to the unstable superoxide anion, which is known to be an important active oxidant in vivo. The purpose of the present study was to clarify the correlation between the superoxide anion and the stable radicals in their reaction with antioxidants.

II Materials and Methods

1. Materials

All chemicals used were purchased. The suppliers were as follows: catechin, quercetin dihydrate, xanthine(X): Sigma Chemical Co.; 3-t-butyl-4- hydroxyanisol (BHA), gallic acid monohydrate, vanillin: Sigma Aldrich Japan; galvinoxyl, caffeic acid, ferulic acid, 3,7-dihydroxyflavone monohydrate, 4-hydroxycoumarin: Aldrich Chem. Co.; catechol, 7,8-dihydroxyflavone, Rutin: Tokyo Kasei; 4-hydroxycoumarin. L-ascorbic acid, hydrochloric acid: Wako Pure Chemical Industries LTD; xanthine oxidase (XO) from Butter milk (0.3 unit/mg): Oriental Yeast Co., LTD.; 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazoloi]-1,3-benzene disulfonate sodium salt (WST-1): Dojindo. Water was purified using YAMATO AutoStill WG25 (18 MΩcm).

2. Method

(1) Experimental procedure

Aqueous sample solutions were dissolved into a mixture of 3 mM xanthine (60 μl), 3 mM EDTA (60 μl), 1.5 mM WST-1 solution (150 μl), and 50 mM sodium carbonate buffer (pH 9.4, 750 μl).

The generation of superoxide anions was initiated by adding 60 μl of XO solution (0.2 mg/ml).

The change in absorbance at 438 nm was monitored over 30 min with a Shimadzu UV-1200 UV-VIS spectrophotometer at room temperature.

(2) Evaluation of relative reaction rate constants

Conventionally, superoxide scavenging capacity has been expressed by the antioxidant concentration that causes a decrease in the generation of the superoxide anion by 50%.

However, the scavenging capacity defined by this method depends on the concentration of the generated superoxide anion. We employed the following kinetic analysis because the result is independent of the concentration of the superoxide anion and has clearer physical meaning.

The kinetic analysis was based on the following reaction scheme:

\[
\begin{align*}
\text{O}_2^- + \text{WST-1} & \rightarrow \text{WST-1}^+ + \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ \\
\text{O}_2^- + \text{AH} & \rightarrow \text{AH}^- + \text{O}_2 \\
\text{O}_2^- + \text{AH} & \rightarrow \text{AH}^- + \text{O}_2
\end{align*}
\]

According to competition kinetics, the MF formation rate (V) corresponds to:

\[
V = \frac{d[MF]}{dt} = \frac{V_0}{2} \left( k_w[WST-1] + k_A[AH] \right)
\]

\(V_0\): the rate of superoxide anion formation from the X/XO system (reaction 1), \(k_w\): the rate constant for the reaction between the superoxide anion and WST-1; \(k_A\): the rate constant for the reaction between the superoxide anion and antioxidant; [WST-1]: the concentration of WST-1; and [AH] = the concentration of the antioxidant.

Since the increase in the absorbance of MF (λmax = 438 nm) was found to be essentially linear to the reaction time during the initial 30 min, the ratio \(V_0/V\) corresponds to \(\Delta A_\omega/\Delta A\), where \(\Delta A_\omega\) and \(\Delta A\) are increases in absorbance at λ = 438 nm in the absence and presence of antioxidants, respectively.

\[
\frac{V_0}{V} = \frac{\Delta A_\omega}{\Delta A} = 1 + \frac{k_A}{k_w}[AH]/[WST-1]
\]

The slope \(k_A/k_w\) calculated from the linear regression of the plot of \([AH]/[WST-1]\) vs. \(V_0/V\), indicated the relative superoxide anion scavenging capacity of each antioxidant.

III Results and Discussion

The kinetic analysis described in the preceding section provides information on the relative scavenging capacity of various compounds toward the superoxide anion. Test samples were chosen from the following criteria: 1. Polyphenols with an o-diphenol ring (including an o,m-triphenol ring), which are known to have high antioxidant capacity. 2. Polyphenols with an o-diphenol ring in which one of the hydroxyl groups is blocked with a methyl group. 3. Well known antioxidants. The
Table 1. Relative reactivity of investigated compounds towards superoxide anion

<table>
<thead>
<tr>
<th>Tested Compound</th>
<th>$k_a/k_w$</th>
<th>$y_0*$</th>
<th>$r^2**$</th>
<th>$N^{***}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>33</td>
<td>0.86</td>
<td>0.999</td>
<td>5</td>
</tr>
<tr>
<td>n-Propyl gallate</td>
<td>30</td>
<td>1.11</td>
<td>0.996</td>
<td>5</td>
</tr>
<tr>
<td>7,8-Dihydroxyflavone</td>
<td>25</td>
<td>0.84</td>
<td>0.964</td>
<td>5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>22</td>
<td>0.99</td>
<td>0.991</td>
<td>5</td>
</tr>
<tr>
<td>Catechin</td>
<td>19</td>
<td>0.99</td>
<td>0.986</td>
<td>5</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>12</td>
<td>0.87</td>
<td>0.997</td>
<td>5</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.2</td>
<td>1.19</td>
<td>0.840</td>
<td>5</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>0.50</td>
<td>1.22</td>
<td>0.973</td>
<td>4</td>
</tr>
<tr>
<td>BHA</td>
<td>0.26</td>
<td>0.95</td>
<td>0.922</td>
<td>5</td>
</tr>
<tr>
<td>Vanillin</td>
<td>inactive at &lt;60 µM</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>4-Hydroxycoumarin</td>
<td>inactive at &lt;60 µM</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>inactive at &lt;400 µM</td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

The ratio $k_a/k_w$ indicates the relative reactivity of substrates towards superoxide anion produced by XO/XOD system.

* intercept
** coefficient of determination
*** number of samples

Some flavonoids (quercetin, myricetin, rutin, and troxerutin) have been shown to inhibit XO oxidation; therefore, they may inhibit the formation of the superoxide anion through reaction (1). If we take this inhibition into consideration, the plot $A_{a}$/
$A_a$ vs [AH]/[WST-1] may deviate from a linear relationship. As shown in Table 1, with the exception of quercetin, the plot gave a good linear relationship. This finding shows that the influence of investigated compounds on xanthine oxidase activity was small, with the exception of quercetin.

In our previous report, we examined the reactivity of various antioxidants with galvinoxyl radicals and DPPH, and reported the relative reactivity as a "Sensitivity factor", which was defined as the slope of a linear regression fitting the data obtained by plotting the degree of decolorization of the galvinoxyl radical or DPPH against the concentration of the added antioxidant. The results are shown in Table 2.

The correlation coefficients between the $k_a/k_w$ values and sensitivity factors for galvinoxyl and DPPH are shown in Table 3. The correlation coefficient was as low as 0.448 (galvinoxyl) or 0.368 (DPPH), and it was shown that the antioxidant capacity assayed by the galvinoxyl or DPPH method did not necessarily reflect superoxide anion scavenging capacity.

A scatter plot for $k_a/k_w$ and sensitivity factor of galvinoxyl is shown in Fig. 2. As shown in Fig. 2, it was very difficult to find any reasonable regularity. The sensitivity factors of antioxidants with an o-diphenol ring or m-triphenol ring with free hydroxyl groups (caffeic acid, 7,8-dihydroxyflavone, gallic acid, catechin, and pyrogallol) were in a fairly narrow range (0.3–0.6), whereas their $k_a/k_w$ values varied widely from 2.2 to 33, and the correlation was rather negative.

Fig. 1. Structures of compounds tested
Table 2. "Sensitivity factor" for galvinoxyl radical and DPPH

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sensitivity factor for Galvinoxyl</th>
<th>Sensitivity factor for DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>0.43</td>
<td>0.036</td>
</tr>
<tr>
<td>7,8-Dihydroxyflavone</td>
<td>0.43</td>
<td>0.044</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.29</td>
<td>0.063</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.56</td>
<td>0.088</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.53</td>
<td>0.051</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.62</td>
<td>0.097</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>0.25</td>
<td>0.025</td>
</tr>
<tr>
<td>BHA</td>
<td>0.09</td>
<td>0.033</td>
</tr>
<tr>
<td>Vanillin</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>4-Hydroxycoumarin</td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.33</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*quoted from reference 9.
"Sensitivity Factor" was defined as the slope of the linear regression fitting to the plot of decolorization (ΔA) of galvinoxyl or DPPH vs. their concentrations.

Table 3. Correlation coefficients among k_A/k_W and sensitivity factors

<table>
<thead>
<tr>
<th>k_A/k_W</th>
<th>Sensitivity Factor for Galvinoxyl</th>
<th>Sensitivity Factor for DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_A/k_W</td>
<td>1</td>
<td>0.448</td>
</tr>
<tr>
<td>Sensitivity Factor for Galvinoxyl</td>
<td>1.000</td>
<td>0.841</td>
</tr>
<tr>
<td>Sensitivity Factor for Galvinoxyl</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Fig. 2. Scatter plot for k_A/k_W and "Sensitivity factor" for galvinoxyl

Although antioxidants with an o- or m-diphenol ring with a hydroxyl group blocked with a methyl group (ferulic acid, BHA, and vanillin) had very low k_A/k_W values, their sensitivity factors were between 0.01 and 0.3, and again no correlation was observed.

Although the superoxide anion radical and galvinoxyl radical are both oxygen radicals, the former is a small, chemically reactive, and negatively charged radical and the latter is a large, stable, and electrically neutral radical. It may be natural for no correlation to exist between the chemical reactivity of these two radicals.

The relationship between k_A/k_W and the sensitivity factor for DPPH was about the same because the correlation coefficient between the two sensitivity factors was high (0.841).

In conclusion, although antioxidant assays using stable radicals attract a lot of attention because of their simplicity, caution should be used when this assay is applied because their reactivity has a small correlation with that of the superoxide anion.

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ノート
抗酸化化物質のスーパーオキシド消去能とガルビノキシル法または
DPPH 法で測定した
抗酸化能との相関
(2012 年 10 月 26 日受理)
(2013 年 1 月 21 日受理)
戸早中美子、落合为一
東亜大学大学院医学科学専攻

キーワード: 抗酸化能、スーパーオキシドアニオン、DPPH、ガルビノキシル、WST-1

概要
11 種の抗酸化性物質（AH）のスーパーオキシドアニオンの消去能を求め、それ等と DPPH およびガルビノキシルラジカルに対する反応性を比較した。スーパーオキシドアニオンはキサンチン/キサンチンオキシダーゼ系で発生させた。スーパーオキシドアニオンのプローブとして4-[(3-(4-ヨードフェニル)-2(4-(4-ヒトロフェニル)-2H-5-テトラゾリオ)-1,3-ベンゼンジルホシ酸 ナトリウム 純 (WST-1) を用い、抗酸化剤の濃度を変化させて共存させた時の WST-1 の発色速度の減少から、以下の式によりスーパーオキシドアノンと WST-1 および抗酸化剤との反応速度の比 (kA/kw) を算出した。kA/kw の値はスーパーオキシドアノン消去能の相対値を表すと考えられる。

\[ R = \frac{\Delta A_0}{\Delta A} = 1 + \frac{k_{d(AH)}}{k_{w(WST-1)}} \]

kA、kw はスーパーオキシドアノンと抗酸化物質および WST-1 との反応速度定数、\( \Delta A_0, \Delta A \) は抗酸化剤が存在しない場合および存在する場合での WST-1 の発色速度を表す。結果はスーパーオキシドアノンの消去能の高い順にコエンザイムQ10、ポリフェノール、7,8-ジヒドロキシフラボン、辣木子酸、アスコルビン酸、ビタミンC、ビタミンE、α-トコフェロール、L-アスコルビン酸、BHA、BHT、ヒドロキシチミン、フェルラ酸であった。kA/kw の値と、これらの抗酸化物質とガルビノキシルラジカルおよび DPPH の以前に報告した抗酸化化物質との相関を求めた。相関係数は 0.448、0.368 と低く、ガルビノキシルラジカル法及び DPPH 法で求めた抗酸化能は必ずしもスーパーオキシドアノンの消去能を反映しないことが示された。