Antioxidant Properties of 2-O-β-D-Glucopyranosyl-L-ascorbic Acid

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The antioxidant activity of a provitamin C agent, 2-O-β-D-glucopyranosyl-L-ascorbic acid (AA-2G), was compared to that of 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and ascorbic acid (AA) using four in vitro methods, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay, 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS\(^{++}\))-scavenging assay, oxygen radical absorbance capacity (ORAC) assay, and 2,2’-azobis(2-aminopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis inhibition assay. AA-2G slowly and continuously scavenged DPPH radicals and ABTS\(^{++}\) in roughly the same reaction profiles as AA-2G, whereas AA quenched these radicals immediately. In the ORAC assay and the hemolysis inhibition assay, AA-2G showed similar overall activities to AA-2G and to AA, although the reactivity of AA-2G against the peroxyl radical generated in both assays was lower than that of AA-2G and AA. These data indicate that AA-2G had roughly the same radical-scavenging properties as AA-2G, and a comprehensive in vitro antioxidant activity of AA-2G appeared to be comparable not only to that of AA-2G but also to that of AA.

Key words: 2-O-β-D-glucopyranosyl-L-ascorbic acid; antioxidant activity; in vitro radical-scavenging assay; moderate and long-lasting radical-scavenging reaction; stable vitamin C derivative

L-Ascorbic acid (AA), known as vitamin C, plays key roles in many biological processes, such as collagen formation, carnitine synthesis, and iron absorption.\(^1,2\) In addition, it is an important antioxidant in food and biological systems,\(^3\) but it is very unstable in aqueous solution. Hence, Yamamoto et al. developed a stable AA derivative, 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G).\(^4-7\) AA-2G is highly stable under the conditions mentioned above, but once it is administered into the body, it is readily hydrolyzed to free AA by mammalian α-glucosidase. Thus AA-2G exhibits inherent vitamin C activity, such as antiscorbutic activity, in vivo, and only AA was detected in the blood in an experiment on oral administration of AA-2G to rats.\(^8-12\) AA-2G has been approved by the Japanese government as a quasi-drug principal ingredient in skin care products and as a food additive.

We have found that AA-2G per se, which had been though to no biological activity, exerted radical-scavenging activity toward unnatural model radicals, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical\(^13-16\) and the 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS\(^{++}\)).\(^15,17\) The chemical properties of AA-2G as a radical scavenger were largely different from those of AA, in that the reaction rate with these model radicals of AA-2G was far slower than that of AA, but the long-term radical scavenging ability per molecule of AA-2G was superior to that of AA under optimal conditions. We also found recently that the radical-scavenging activity of AA-2G was biological relevant using a cell-based antioxidant assay system, 2,2’-azobis(2-aminopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis inhibition assay.\(^18\) These data suggest that AA-2G can be used not only as a stable source of AA but also as a useful antioxidant in the food and cosmetics fields.

Recently, a stereoisomer of AA-2G, 2-O-β-D-glucopyranosyl-L-ascorbic acid (AA-2G), was isolated from lycheum fruit, a popular traditional Chinese food.\(^19\) The chemical structures of AA, AA-2G, and AA-2βG are shown in Fig. 1. AA-2βG is also a stable AA derivative, and has been found to have vitamin C activity using osteogenic disorder Shionogi (ODS) rats.\(^20\) Differently from AA-2G, it was reported that oral administration of

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AA-2βG to rats increased the level of AA-2βG as well as AA in the blood of the portal vein, probably due to lesser distribution of β-glucosidase than of α-glucosidase.19) Thus, if AA-2βG per se possesses antioxidant activity as dose AA-2G, it might contribute to antioxidant defense in the body. As far as we know, there have been no reports on the antioxidant activities of AA-2βG. Hence in this study we investigated the antioxidant activity of AA-2βG per se in vitro, and compared it to those of AA-2G and AA. Two methods utilizing unnatural model radicals, DPPH radical-scavenging assay and ABTS⁺⁺-scavenging assay, and two methods utilizing a peroxyl radical, oxygen radical absorbance capacity (ORAC) assay and AAPH-induced erythrocyte hemolysis inhibition assay, were employed.

Materials and Methods

Chemicals. AA, sodium fluorescein, and AAPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). AA-2G and AA-2βG were gifts from Hayashibara Biochemical Laboratories (Okayama, Japan) and Suntory (Osaka, Japan) respectively. DPPH (100 μM) was from Sigma Chemical (St. Louis, MO). H₂O₂ (30%) was from Nacalai Tesque (Kyoto, Japan). Sheep erythrocytes were obtained from Nihon Seibutsu Zairyou Center (Tokyo). Reagents from Nihon Seibutsu Zairyou Center (Tokyo). Reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Horikawa Chemical Industry (Okayama, Japan) and Suntory (Osaka, Japan) respectively. DPPH was from Sigma Chemical (St. Louis, MO). H₂O₂ (30%) was from Nacalai Tesque (Kyoto, Japan). Sheep erythrocytes were obtained from Nihon Seibutsu Zairyou Center (Tokyo). Reagents were used without further purification. All water used was Milli-Q grade.

DPPH radical-scavenging assay. The radical-scavenging activities of AA-2βG, AA-2G, and AA against DPPH radical were assessed as described in previous papers.14,15) Briefly, DPPH (100 μM) was mixed with an antioxidant (20 μM) in 60% ethanol/40% citric acid-sodium citrate buffer (10 mM, pH 3–6). The reaction was carried out under an atmosphere of argon at 25 °C. Changes in the absorbance at 524 nm due to scavenging of DPPH radicals and ABTS⁺⁺ concentration were measured with a spectrophotometer.

ABTS⁺⁺-scavenging assay. The radical-scavenging activities of AA-2βG, AA-2G, and AA against ABTS⁺⁺ were assessed as described in a previous paper.17) Briefly, ABTS⁺⁺ (100 μM) generated with an ABTS/H₂O₂/HRP system was mixed with an antioxidant (20 μM) in citric acid-sodium citrate buffer (50 mM, pH 3–6). The reaction was carried out under an atmosphere of argon at 25 °C. Changes in the absorbance at 730 nm due to the scavenging of ABTS⁺⁺ were measured with a spectrophotometer.

Stoichiometric studies for DPPH radical-scavenging assay and ABTS⁺⁺-scavenging assay. The numbers of DPPH radicals and ABTS⁺⁺ scavenged by each antioxidant were calculated by following equation:

\[
RSA(n) = \frac{\Delta A_{120}/A_0}{\text{radical}/\text{antioxidant}}
\]

where RSA(n) is radical-scavenging activity factor n in moles of radicals scavenged by each mol of antioxidant; \(\Delta A_{120}\) is the absorbance difference between the reaction solution and control at 120 min; \(A_0\) is the initial absorbance of the control; [radical] is the DPPH radical antioxidant concentration (100 μM); and [antioxidant] is the AA-2βG, AA-2G, and AA concentration (20 μM).

ORAC assay. ORAC assay for AA-2βG, AA-2G, and AA was carried out as described by Gillespie et al.,21) with a slight modification. Briefly, fluorescein (60 μM), antioxidant (12.5 μM), and AAPH (18.75 μM) were incubated in 200 μM of KH₂PO₄-K₂HPO₄ buffer (75 mM, pH 7.0) at 37 °C in a 96-well plate (Costar no. 3615). The fluorescence (an excitation wavelength of 485 nm and an emission wavelength of 520 nm) was monitored every 1 min for 1 h by Powerscan HT (DS Pharma Biomedical, Osaka, Japan). The area under the curve (AUC) was calculated as

\[
AUC = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \cdots + f_{38}/f_0 + f_9/f_0
\]

where \(f_0\) is the initial fluorescence reading at 0 min and \(f_i\) is the fluorescence reading at i min. The net AUC was obtained by subtracting the AUC of the blank from that of a sample.

AAPH-Induced erythrocyte hemolysis inhibition assay. Sheep erythrocytes were washed 2 times with phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na₂HPO₄, and 1.9 mM NaH₂PO₄, pH 7.4), and resuspended in PBS at a 10% (v/v) suspension. The erythrocyte suspension (1.6 ml) and an antioxidant in PBS (125 μM, 12.8 ml) were mixed into a flat-bottomed
test tube (30 × 120 mm), and incubated in a water bath shaker (Taitec, Saitama, Japan) at 37 °C for 5 min. An ice-cold AAPH/PBS solution (400 mM, 1.6 ml) was injected into the above mixture to initiate hemolysis. An aliquot of this mixture was periodically withdrawn and centrifuged, and the absorbance at 524 nm of the supernatant was measured with a spectrophotometer. The degree of hemolysis (%) was determined from the concentrations of hemoglobin in the supernatant. The value of 100% hemolysis was determined from the supernatant by adding 9 volumes of water to 1 volume of 10% (v/v) erythrocytes.

**Results**

**DPPH radical-scavenging assay and ABTS**++-scavenging assay

The DPPH radical is a relatively stable radical that does not exist in nature. The deep purple color of the DPPH radical disappears when the radical is reduced, and so decreases in the radical can easily be monitored with a spectrometer.22) Hence DPPH assay is one of the most common methods of assessing antioxidant activities. ABTS**++ is also a relatively stable unnatural radical. It bears a blue-green color, and decoloration assay can be performed in a manner similar to DPPH assay.22)

A time-course study of the DPPH radical-scavenging reactions of AA-2βG, AA-2G, and AA was carried out in 60% ethanol/40% citrate buffer (pH 3) for 2 h (Fig. 2), since AA-2G reacted with the DPPH radical more effectively at pH 3 than at pH 6, as reported in previous papers.14,15) AA-2βG showed the same reaction curve as AA-2G. AA-2βG and AA-2G continuously reacted with the DPPH radical for 2 h, whereas AA rapidly scavenged the DPPH radical, within 1 min.

![Fig. 2. Time Course of DPPH Radical-Scavenging Reaction of AA-2βG, AA-2G, and AA.](image)

AA-2βG, AA-2G, or AA (20 μM) and the DPPH radical (100 μM) were incubated in 60% ethanol/40% citrate buffer (10 mM, pH 3) at 25 °C. Changes in absorbance at 524 nm due to scavenging of the DPPH radical were monitored at the indicated times. Values are means ± S.D. of three separate experiments. Absence of S.D. bar means that the S.D. bar is within the symbol.

The reactions of AA-2βG, AA-2G, and AA toward ABTS**++ were measured in citrate buffer (pH 6) for 2 h (Fig. 3), since AA-2G was found to react with ABTS**++ more effectively at pH 6 than at pH 3.15,17) AA-2βG scavenged the ABTS**++ in a manner similar to AA-2G. The reaction of these two AA derivatives proceeded for 2 h, whereas the reaction of AA was completed within 1 min.

We have found that the reactivity of AA-2G against the DPPH radical is largely affected by the pH of the reaction mixture.14,15) Hence the effects of pH on the long-term reaction stoichiometry of AA-2βG in the DPPH radical- and ABTS**++-scavenging assays were compared to those of AA-2G and AA (Table 1). The stoichiometric factor RSA(n) was defined as the number of radicals consumed per molecule of each antioxidant in a 2-h reaction. AA-2βG showed almost the same profiles as AA-2G. The RSA(n) of these two AA derivatives against the DPPH radical in a pH range of 3 to 4 was greater than that of AA, while their scavenging abilities sharply decreased with increasing pH, and disappeared at pH 6. On the other hand, their RSA(n) values against ABTS**++ were little affected by pH changes, and were superior to that of AA at all pH levels. The RSA(n) values of AA were about 2 in all cases examined.

**ORAC assay and AAPH-induced erythrocyte hemolysis inhibition assay**

ORAC assay and AAPH-induced erythrocyte hemolysis inhibition assay utilize a peroxyl radical, which is involved in biologically relevant oxidative processes. ORAC assay measures the inhibition of peroxyl radical-induced oxidations of a fluorescent probe by antioxidants.22) The peroxyl radical is continuously generated by thermal degradation of AAPH, and it reacts with...
Antioxidant Properties of AA-2βG

Table 1. Effects of pH on RSA(n) of AA-2βG, AA-2G, and AA against the DPPH Radical and ABTS**^+

<table>
<thead>
<tr>
<th>pH</th>
<th>RSA(n) against DPPH radical</th>
<th>RSA(n) against ABTS**^+</th>
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<tbody>
<tr>
<td></td>
<td>AA-2βG</td>
<td>AA-2G</td>
</tr>
<tr>
<td>pH 3</td>
<td>2.7 ± 0.0</td>
<td>2.1 ± 0.0</td>
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<tr>
<td>pH 4</td>
<td>2.2 ± 0.0</td>
<td>2.1 ± 0.0</td>
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<tr>
<td>pH 5</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.0</td>
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<tr>
<td>pH 6</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
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In the case against the DPPH radical, the reaction mixture contained AA-2βG, AA-2G, or AA (20 μM) and the DPPH radical (100 μM) in 5.0 ml of 60% ethanol/40% citrate buffer (10 mM, pH 3–6). In the case against ABTS**, the reaction mixture contained AA-2βG, AA-2G, or AA (20 μM) and ABTS**^+ (100 μM) in 5.0 ml of citrate buffer (50 mM, pH 3–6). The reaction was carried out at 25 °C for 2 h. The number of radicals scavenged per molecule of AA derivatives and of AA is expressed as RSA(n). Results are means ± S.D. (n = 6).

In the ORAC assay, inhibition of fluorescence decay by AA-2βG showed quite different profiles from that by AA-2G and AA (Fig. 4). In the early phase of the reaction, AA-2βG and AA-2G partially suppressed the loss of fluorescence, whereas AA completely inhibited it. The initial inhibition effects were in the order AA > AA-2G > AA-2βG. In the case of AA, fluorescence rapidly decayed after a lag time of about 30 min. On the other hand, AA-2G continued partial inhibition for a longer period. Furthermore, AA-2βG maintained partial inhibition during the whole period. As a result, the remaining fluorescence intensity was reversed in the late phase of the reaction in the order AA-2βG > AA-2G > AA. In this way, the profiles of the fluorescence decay curves of AA-2βG, AA-2G, and AA were largely different. On the other hand, in the ORAC assay, the total extent of inhibition by an antioxidant is expressed by net AUC, which is obtained by subtracting the area under the blank curve from the area under the sample curve. The net AUCs of AA-2βG, AA-2G, and AA were 31.9 ± 0.2, 37.6 ± 0.6, and 31.6 ± 0.7 respectively (means ± S.D. of triplicate experiments). Thus, the overall antioxidant activity of AA-2βG in the ORAC assay was roughly in the same range as that of AA-2G and AA. In the AAPH-induced erythrocyte hemolysis inhibition assay, AA-2βG also inhibited hemolysis to the same extent as AA-2G and AA (Fig. 5).

Discussion

In the present study, we investigated the antioxidant activity of AA-2βG per se in comparison to those of AA-2G and AA using four in vitro methods. In assays utilizing unnatural model radicals, the DPPH radical-scavenging assay and the ABTS**^+ -scavenging assay, AA-2βG showed reaction properties similar to AA-2G, i.e., slow and long-lasting radical-scavenging as compared to AA (Figs. 2, 3). The effects of pH on the RSA(n) values were also nearly the same as between AA-2βG and AA-2G (Table 1). In addition, the RSA(n) values of these AA derivatives exceeded that of AA under optimal conditions. In the ORAC assay, which utilizes a similar kind of peroxy radical actually generated in vivo, the profiles of the fluorescence decay curves of AA-2βG, AA-2G, and AA were quite different (Fig. 4), but their net AUC, which reflects overall antioxidant activity in the ORAC assay, were roughly in the same range. In the AAPH-induced erythrocyte hemolysis inhibition assay, AA-2βG showed quite different profiles from that of AA-2G and AA (Fig. 5).
hemolysis inhibition assay, a cell-based antioxidant assay utilizing the same radical as in the ORAC assay, AA-2βG showed almost the same inhibition efficacy as AA-2G and AA (Fig. 5). Therefore, AA-2βG had generally similar characteristics to AA-2G in the in vitro antioxidant assays, except for the ORAC assay.

Differences between AA-2βG and AA-2G were clearly observed in the ORAC assay (Fig. 4). Initial inhibition efficacy due to AA-2βG was inferior to that due to AA-2G, indicating that AA-2βG was less reactive against the AAPH-derived peroxyl radical than AA-2G. In addition, in the ABTS**+-scavenging assay, AA-2βG and AA-2G also exhibited slight but significant differences (p < 0.001, unpaired t-test), i.e., the amounts of ABTS**+ quenched by AA-2βG were lower than those by AA-2G in the early phases of the reaction (1 and 5 min, Fig. 3), and the RSA(n) values of AA-2βG at pH levels 3 and 4 were also lower than those of AA-2G (Table 1). These results are attributable to their stereochemical differences at the C-2 position, the α-glucosidic bond or the β-glucosidic bond (Fig. 1). In this way, the reactivity of AA-2βG toward certain radicals might be inferior to that of AA-2G. However, the net AUC in the ORAC assay (Fig. 4) and the amount of finally quenched ABTS**+ (120 min, Fig. 3) were similar as between AA-2βG and AA-2G. Thus their in vitro antioxidant activity appeared not to be greatly different.

In AA-2βG and AA-2G the oxidizable hydroxyl group at C-2 position of AA moiety was replaced with the glucosyl group (Fig. 1), and thus AA-2βG and AA-2G can be expected to exert lower radical-scavenging activity than AA. It is true that the reactivity of these AA derivatives toward radicals was always inferior to that of AA (Figs. 2–4), but their reaction stoichiometry against unnatural model radicals was occasionally superior to that of AA (Table 1), and their net AUC in the ORAC assay was comparable to that of AA (Fig. 4). Furthermore, AA-2βG and AA-2G protected erythrocytes from biologically relevant peroxyl radicals to the same extent as AA (Fig. 5). These findings suggest that these AA derivatives act as potent antioxidants comparable to AA in the biological context. Recently, we found that AA-2G scavenged the DPPH radical not via AA as an intermediate but via a covalent adduct formation with the radical.10 This unique reaction was the reason AA-2G showed unexpectedly high reaction stoichiometry against the DPPH radical. The results obtained in this study suggest that AA-2βG possesses essentially the same radical-scavenging mechanisms as AA-2G, and we believe that the potent antioxidant activities of AA-2G and AA-2βG comparable to that of AA can be explained by a covalent adduct formation with a radical.

In conclusion, AA-2βG had roughly the same radical-scavenging properties as AA-2G, and the comprehensive in vitro antioxidant activity of AA-2βG appeared to be comparable not only to that of AA-2G but also to that of AA. Recently, it was reported that AA-2G and another AA derivative, ascorbic acid 2-phosphate, can protect the skin from UVB/A-induced photodamage ex vivo by themselves more effectively than AA.25 Hence it may be necessary to consider the antioxidant activities of AA derivatives per se not only in vitro but also in vivo. As mentioned in the introduction, in vivo, the distribution of β-glucosidase, which hydrolyzes AA-2βG to AA, is limited as compared to that of α-glucosidase, which converts AA-2G to AA. Thus AA-2βG is more likely to remain in an unmetabolized form than AA-2G, and so the antioxidant activity of the unmetabolized AA derivatives per se may be more important to AA-2βG than to AA-2G in vivo.

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17) Takebayashi, J., Tai, A., and Yamamoto, I., pH-dependent long-term radical scavenging activity of AA-2G and 6-Octa-AA-2G against 2,2′-azinobis(3-ethylbenzothia-