

Stabilizing Effect of Grape Seed Extract on Ascorbic Acid

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L-Ascorbic acid (AsA) has numerous biological activities. It is known that AsA is unstable under neutral and alkaline conditions, degrading almost completely within several hours, whereas it is relatively stable under acidic conditions. The present study investigated the effect of grape seed extract (GSE), which contains proanthocyanidins, on the stability of AsA under neutral and alkaline conditions. The addition of GSE to AsA solution in 3,3'-dimethylglutaric acid-tris(hydroxymethyl)aminomethane-2-amino-2-methyl-1,3-propanediol (GTA) (50 mM, pH 7.0 or 10.0) buffer significantly increased the remaining amount of AsA and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, compared with those of AsA alone. In particular, it was clarified that GSE contributed to the stability of AsA at an alkaline pH. We also investigated the effect of GSE on the stability of AsA under *quasi*-physiological conditions. It was revealed that GSE stabilized AsA in simulated intestinal juice (pH 8.5) at 37°C. DPPH radical-scavenging activity was closely correlated with the remaining amounts of AsA. The present results, although not directly transferable to *in vivo* conditions, suggest that GSE may stabilize AsA under neutral and alkaline conditions and affect the physiological activity of AsA.

Keywords: grape, proanthocyanidin, (+)-catechin, ascorbic acid, antioxidation, radical-scavenging activity

Introduction

L-Ascorbic acid (AsA, the reduced form of vitamin C) has been reported to have a wide range of biological properties, including increasing nitric oxide synthesis, lowering the rate of low-density lipoprotein oxidation, inhibiting platelet activation, stimulating the production of anti-inflammatory cytokines, and inhibiting the production of certain proinflammatory cytokines (Carr and Frei, 1999). These diverse biological activities are thought to be attributable to the structure of this chemical. However, it is known that AsA is unstable under neutral and alkaline conditions.

Grape seed extract (GSE) contains proanthocyanidins, which are oligomers or polymers of polyhydroxy flavan-3-ol units. Proanthocyanidins have remarkable radical-scavenging activities, especially for hydrophilic radicals (Ariga and Hamano, 1990), and inhibit LDL oxidation *in vitro* (Teissedre *et al.*, 1996). Therefore, dietary proanthocyanidins may reduce the incidence of heart diseases such as arteriosclerosis (Yamakoshi *et al.*, 1999). We previously demonstrated that GSE is more stable than AsA under conditions of neutral or alkaline pH (Kitao *et*

al., 2001). Therefore, it is likely that GSE contributes to the stability of AsA, especially under neutral and alkaline conditions, when GSE is added to AsA solution.

It has been reported that AsA contributes to the stability and antioxidative activity of tocopherols (vitamin E) (Niki *et al.*, 1985, Doba *et al.*, 1985), green tea catechin (Chen *et al.*, 1998) and cocoa polyphenols (Zhu *et al.*, 2003). Although AsA was used as a supplementary material to maintain the effect of catechins and polyphenols in these cases, AsA itself is degraded in a short time. We focused on the importance of maintaining the stability of AsA because it is widely used in the food industry as an ingredient and antioxidant. To this end, we examined the stabilizing effect of GSE on AsA under various conditions, including physiologically realistic pH values.

Materials and Methods

Materials A proanthocyanidin-rich extract of GSE was prepared from grape seeds (*Vitis vinifera* L.) (Ariga *et al.*, 2000). Briefly, grape seeds were washed with water at 60°C for 2 h and then extracted with water at 90°C for 2 h. The aqueous extract was freeze-dried to obtain a proanthocyanidin-rich extract. The extract was composed of 73.4% proanthocyanidins, 5.6% monomeric flavanols, 6.4% organic acids, 3.9% ash, 3.7% protein, 3.0% moisture, and 1.7% carbohydrate. AsA, 1,1-diphenyl-2-picrylhydrazyl (DPPH), tris(hydroxymethyl)aminomethane (Tris), 2,4-dinitrophenylhydrazine, 2,6-dichloroindophenol, and acetonitrile (HPLC grade) were purchased from Nacalai

Abbreviations: AsA, L-ascorbic acid; GSE, grape seed extract; DPPH, 1,1-diphenyl-2-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; GTA, 3,3'-dimethylglutaric acid-tris(hydroxymethyl)aminomethane-2-amino-2-methyl-1,3-propanediol.

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Tesque Inc. (Kyoto). 3,3-Dimethylglutaric acid and 2-amino-2-methyl-1,3-propanediol were obtained from Wako Pure Chemical Industries (Osaka), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Aldrich Chemical Co. (Milwaukee, WI). Other chemicals used were of highest grade commercially available. The water used in this experiment was purified with Milli-Q Labo equipment (Millipore Japan, Tokyo).

Heat Treatment The stability of AsA (50 mg/l) was assessed in each buffer or juice in the presence or absence of GSE (50 mg/l). Each sample was dissolved in 50 mM 3,3-dimethylglutaric acid-tris(hydroxymethyl)aminomethane-2-amino-2-methyl-1,3-propanediol (GTA) buffer (pH 4.0, 7.0, or 10.0) or a simulated gastric juice (0.24% hydrochloric acid-0.2% sodium chloride solution, pH 1.8) or intestinal juice (1.5% sodium hydrogen carbonate solution, pH 8.5) (Yoshino *et al.*, 1999). Aliquots of each sample solution (1.0 ml) were placed in screw-capped vials and heated in an air bath at the indicated temperature and for the indicated time. "GSE alone" and "AsA alone" denote the results for either GSE or AsA, respectively, incubated alone. "GSE plus AsA" denotes the result of GSE alone added to the result of AsA alone. "Premixed GSE with AsA" denotes the result obtained when GSE and AsA were mixed before incubation at each pH for the indicated time in the same vessel. Each incubated sample was tested for AsA content (by HPLC) and DPPH radical-scavenging activity.

Analysis of AsA by reverse-phase HPLC AsA content was determined by HPLC according to a modified method of Kishida *et al.* (1992). Briefly, 100 μ l of AsA solution or 100 μ l of AsA solution mixed with 0.2% (w/v) 2,6-dichloroindophenol (50 μ l) was added to 1.0% (w/v) stannous chloride in 5.0% (w/v) metaphosphoric acid solution (50 μ l) and 2.0% (w/v) 2,4-dinitrophenylhydrazine (120 μ l) in 4.5 mol/l sulfuric acid. The resultant mixture was incubated for 3 h at 37°C. Ethyl acetate (1.0 ml) and water (1.0 ml) were added to the reaction mixture. After shaking and centrifugation (1500 \times g, 4°C) for 5 min, 300 μ l of the ethyl acetate layer was removed and dried under nitrogen gas. The resulting residue was dissolved in acetonitrile (200 μ l) and applied to HPLC.

HPLC analysis was done under the following conditions: column, CAPCELL PAK C₁₈ UG120 (4.6 \times 250 mm) (Shiseido Co., Ltd., Tokyo); detection, 505 nm; column temperature, ambient temperature; mobile phase, 50% (v/v) acetonitrile adjusted to pH 3.5 with 0.1% (v/v) triethylamine and phosphoric acid; applied volume, 20 μ l; and flow rate, 1.0 ml/min. Identification of AsA was confirmed by comparison of retention time and co-elution with the authentic standard. Quantitative determination of AsA was based on the external standard method. A standard curve was constructed using standard solutions of AsA with the same HPLC protocol. AsA content was calculated from the difference between the values obtained for the samples with and without 2,6-dichloroindophenol.

Measurement of DPPH radical-scavenging activity Radical-scavenging activity was determined by the DPPH

(Blois, 1958)-colorimetric method (Yamaguchi *et al.*, 1998). In brief, an aliquot of the sample solution (200 μ l) was mixed with 100 mM Tris-HCl buffer (pH 7.4, 800 μ l) and added to 1.0 ml of 500 μ M DPPH in ethanol (for a final concentration of 250 μ M). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the reaction mixture at 517 nm was then measured. A blank which did not contain the sample solution was measured for comparison. DPPH radical-scavenging activity was evaluated by calculating the difference in DPPH radical absorbance, detected at 517 nm, between a blank and a sample. Trolox was used as a control standard. The data are expressed as μ mol of Trolox equivalent, because Trolox is a stable antioxidant and is widely used as an index of antioxidative activity.

Statistical methods Statistical significance was evaluated using Student's *t*-test. $P < 0.05$ was considered to be statistically significant. Microsoft Excel was used for all linear regressions. The significance of *R* values was determined using a table for the critical values of the Pearson's product moment correlation coefficient (O'Mahony, 1986).

Results and Discussion

Effect of GSE on the stability of AsA in GTA buffer

Figure 1 shows the remaining amounts of AsA in 50 mM GTA buffer (pH 4.0, 7.0, or 10.0) after 30 min incubation at various temperatures. "Premixed GSE with AsA" denotes the remaining amount of AsA when GSE and AsA were pre-mixed in the same vessel before incubation at each pH for the indicated time. AsA was unstable under neutral and alkaline conditions, although it was relatively stable at pH 4.0. When AsA was incubated at 100°C in GTA buffer (pH 7.0), more than 50% of AsA was degraded within 30 min; when incubated at 100°C in GTA buffer (pH 10.0) for 30 min, AsA disappeared completely. Even at 0°C, about 40% of AsA was degraded.

The stability of AsA in the presence of GSE (50 mg/l) was also examined in the same buffers. After incubation at pH 4.0, the remaining amount of AsA was almost the same as that of AsA alone, which suggests that AsA is stable at acidic pH. It was found that the addition of GSE significantly improved the stability of AsA under neutral and alkaline conditions. About 1.2 times the amount of AsA found in AsA alone remained after incubation at every temperature in the presence of GSE at a neutral pH (pH 7.0), although the concentrations gradually decreased as the temperature rose (Fig. 1). Even at alkaline pH (pH 10.0) and over 75°C, significant amounts of AsA remained after incubation when GSE was added, whereas all (or nearly all) AsA disappeared when AsA was incubated alone. Thus, GSE afforded effective protection for AsA under these conditions, especially in neutral to alkaline conditions.

Effect of GSE on radical-scavenging activity of AsA in GTA buffer Figure 2 shows the DPPH radical-scavenging activities of GSE alone, AsA alone, GSE plus AsA, and premixed GSE with AsA in 50 mM GTA buffer (pH 4.0, 7.0,

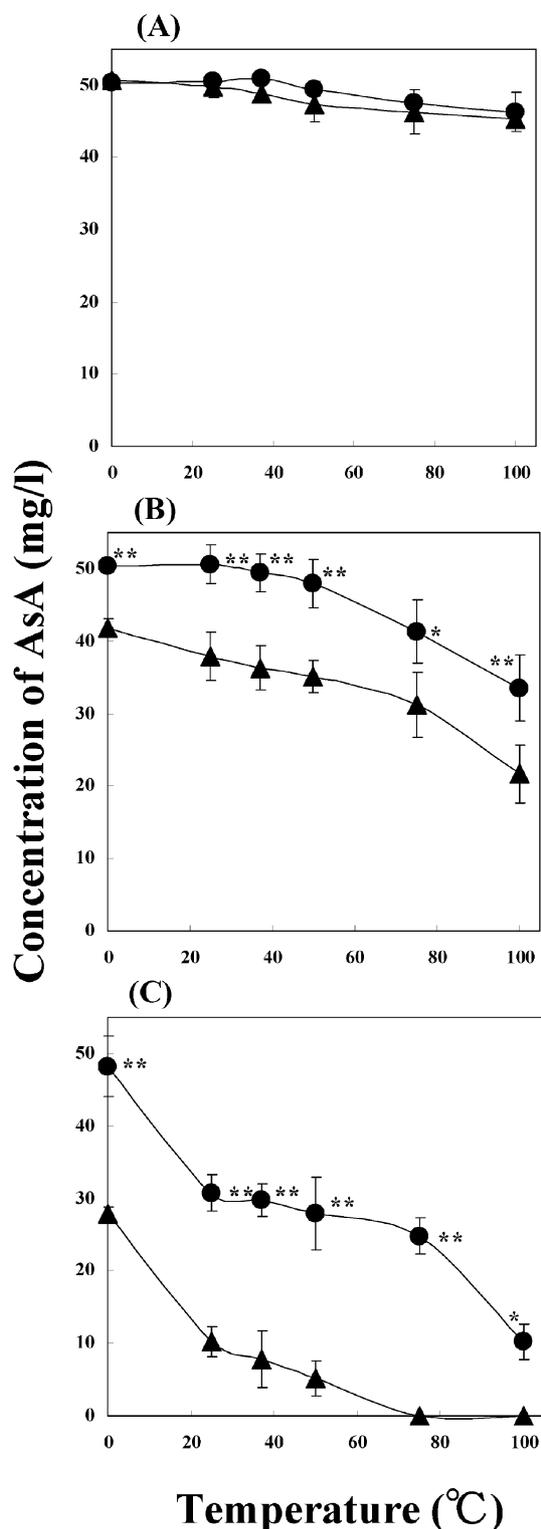


Fig. 1. Remaining amounts of AsA in GTA buffer solutions at various temperatures.

Each sample solution containing 50 mg/l of AsA was incubated for 30 min in 50 mM GTA buffer (pH 4.0-A, 7.0-B, or 10.0-C) at various temperatures. The remaining amounts of AsA were measured by the dinitrophenylhydrazine-HPLC method. Each value is the mean \pm standard deviation of five different replicates. ▲: AsA alone, ●: premixed GSE with AsA. *: $p < 0.05$, **: $p < 0.01$ vs. AsA alone.

or 10.0) after incubation for 30 min at various temperatures. The mean of GSE plus AsA is the value of the result of GSE alone added to the result of AsA alone. "Premixed GSE with AsA" denotes the remaining radical-scavenging activity when GSE and AsA were mixed in the same vessel before incubation at each pH for the indicated time. We decided that if the value of premixed GSE with AsA was significantly higher than that of GSE plus AsA, the addition of GSE could be said to have a synergistic effect on the radical-scavenging activity of AsA.

The radical-scavenging activity of premixed GSE with AsA was almost the same as that of GSE plus AsA at pH 4.0. Both AsA and GSE are comparatively stable under acidic conditions; thus, premixing of GSE with AsA results in simple addition of their effects. At pH 7.0, the radical-scavenging activity of premixed GSE with AsA was found to be significantly higher than that of GSE plus AsA when the incubation temperature was over 50°C. At pH 10.0, when the incubation temperature was over 37°C, the radical-scavenging activity of premixed GSE with AsA was significantly higher than that of GSE plus AsA. These results suggest that a synergistic effect on the radical-scavenging activity of GSE and AsA was obtained when GSE was premixed with AsA and the resulting solution was incubated at comparatively high temperatures under neutral to alkaline conditions.

The correlation between the remaining amounts of AsA and DPPH radical-scavenging activity at pH 4.0, 7.0, and 10.0 for both AsA alone and premixed GSE with AsA were examined (Fig. 3). It was revealed that DPPH radical-scavenging activity was closely correlated with the concentration (remaining amount) of AsA at pH 7.0 and 10.0 but not at pH 4.0. It is thought that the addition of GSE protected AsA from decomposition under neutral to alkaline conditions, and accordingly, AsA retained its radical-scavenging activity.

Effect of GSE on the stability of AsA in simulated gastric and intestinal juices Figure 4 shows the change in the remaining amounts of AsA under quasi-physiological conditions at 37°C. In the case of simulated gastric juice (pH 1.8), the time courses of AsA decomposition were almost the same for AsA alone and premixed GSE with AsA; more than 90% of AsA remained after incubation for 6 h. In contrast, in simulated intestinal juice (pH 8.5), AsA was degraded rapidly during incubation. In the case of AsA alone, AsA disappeared completely after incubation for 4 h, whereas about 10% of the original amount of AsA remained in the presence of GSE. When GSE was present, the amounts of AsA remaining were significantly higher than those of AsA alone when the incubation time was over 1 h. It seems that GSE may contribute to the stability of AsA in simulated intestinal juice.

Effect of GSE on radical-scavenging activity of AsA in simulated gastric and intestinal juices The time courses of the radical-scavenging activity of GSE alone, AsA alone, GSE plus AsA, and premixed GSE with AsA under quasi-physiological conditions at 37°C were examined

(Fig. 5). The radical-scavenging activities of GSE alone and AsA alone were almost constant in simulated gastric juice, although that of AsA alone decreased slightly. Consequently, the radical-scavenging activity of GSE plus AsA was almost constant. No synergistic effect on the DPPH radical-scavenging activity of GSE and AsA was obtained because the radical-scavenging activities of both chemicals are stable under acidic conditions. It was

expected that AsA might be more stable in simulated gastric juice (pH 1.8) than GTA buffer (pH 4.0) due to the greater acidity of the former.

In the case of simulated intestinal juice, the radical-scavenging activity of AsA alone decreased rapidly. Even after an incubation time of 0.5 h, less than 50% of radical-scavenging activity remained compared with that in simulated gastric juice. After incubation for 6 h, the

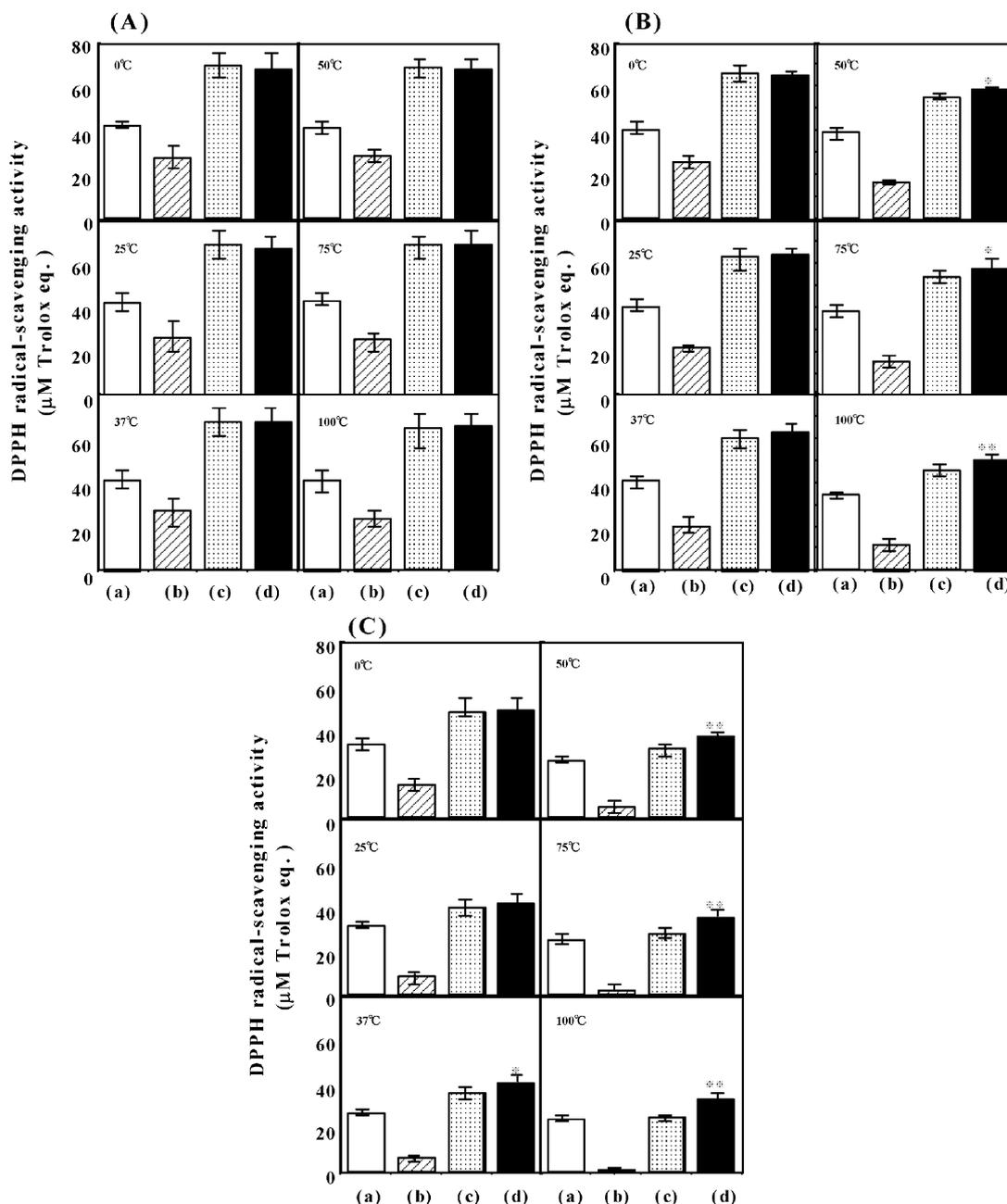


Fig. 2. Remaining DPPH radical-scavenging activity in GTA buffer solutions at various temperatures.

Each sample solution (50 mg/l) was incubated in 50 mM GTA buffer (pH 4.0 (A), 7.0 (B), or 10.0 (C)) for 30 min at various temperatures, and the remaining DPPH radical-scavenging activity was measured by the colorimetric method. Each value is the mean \pm standard deviation of five different replicates. (a): GSE alone, (b): AsA alone, (c): GSE plus AsA, (d): premixed GSE with AsA. *: $p < 0.05$, **: $p < 0.01$ vs. GSE plus AsA.

radical-scavenging activity of AsA alone disappeared completely. In contrast, the radical-scavenging activity of GSE alone was almost constant, although it decreased slightly after incubation for 6 h. The radical-scavenging activity of GSE plus AsA gradually decreased, which reflected the result of AsA alone. Compared with the value of GSE plus AsA, that of premixed GSE with AsA was significantly higher at every time except for 0 h.

Similarly to Fig. 2, a synergistic effect on the radical-scavenging activity of GSE and AsA was obtained. It seems that GSE may contribute to the radical-scavenging activity of AsA as well as its stability under alkaline conditions such as simulated intestinal juice.

The correlation between the remaining amount of AsA and DPPH radical-scavenging activity under *quasi*-physiological conditions is shown in Fig. 6. It can be

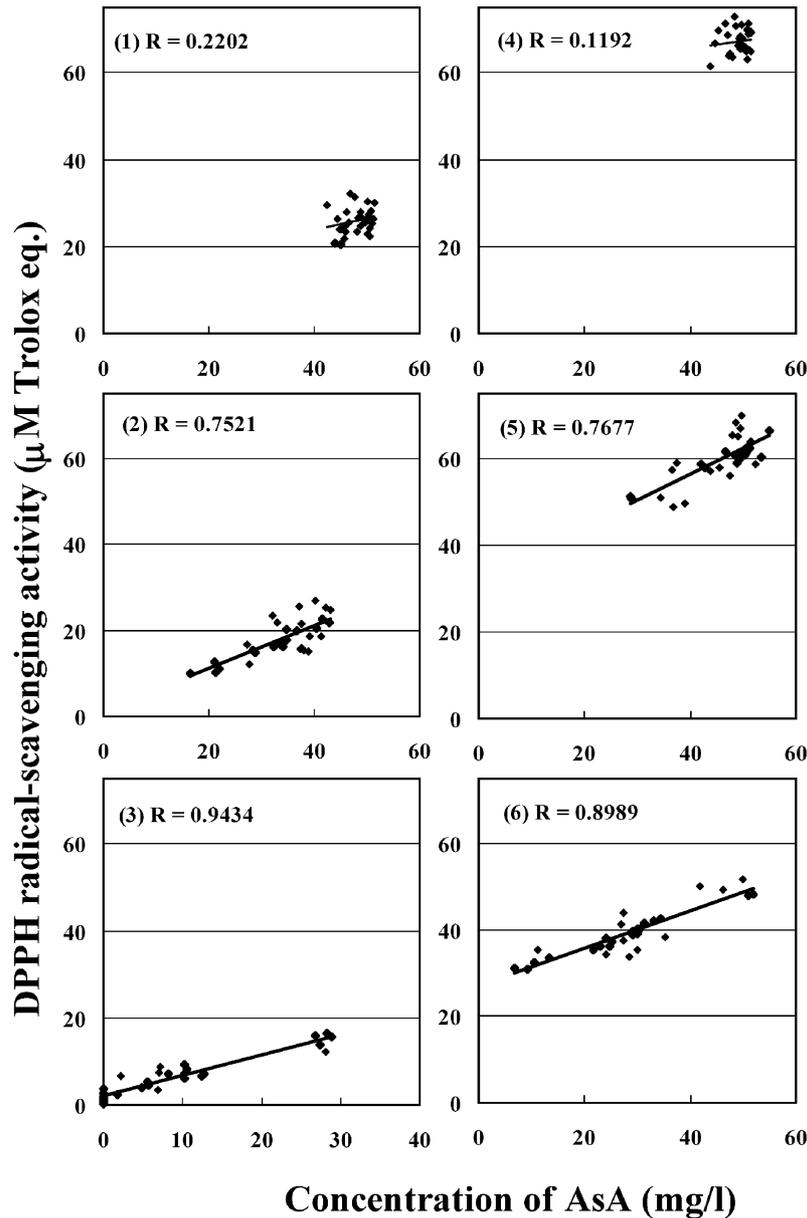


Fig. 3. Correlation between the remaining amounts of AsA and DPPH radical-scavenging activity in GTA buffer. (1): AsA alone in 50 mM GTA buffer (pH 4.0), (2): AsA alone in 50 mM GTA buffer (pH 7.0), (3): AsA alone in 50 mM GTA buffer (pH 10.0), (4) premixed GSE with AsA in 50 mM GTA buffer (pH 4.0), (5): premixed GSE with AsA in 50 mM GTA buffer (pH 7.0), (6): premixed GSE with AsA in 50 mM GTA buffer (pH 10.0).

seen that radical-scavenging activity was closely correlated with the concentration (remaining amount) of AsA in simulated intestinal juice as well as the results using GTA buffer solutions (Fig. 3). In order to maintain its physiological functions, such as radical-scavenging activity, it is important to find ways to protect AsA from degradation. GSE was chosen for use in this study because of its water-solubility and safety. We suggest that, when incubated together with AsA, GSE serves as a reductant that protects ascorbic acid from degradation.

The present results, although not directly transferable

to *in vivo* conditions, may have implications for the problem of degradation of AsA before absorption. Given that intestinal pH is neutral to slightly alkaline, it is reasonable to speculate that the concurrent presence of GSE may partially prevent degradation of AsA in the intestine before absorption. The influence of dietary GSE on the absorption of AsA deserves further study. The present study also suggests that a different type of stable antioxidant such as GSE can significantly influence the stability of AsA in foods and beverages.

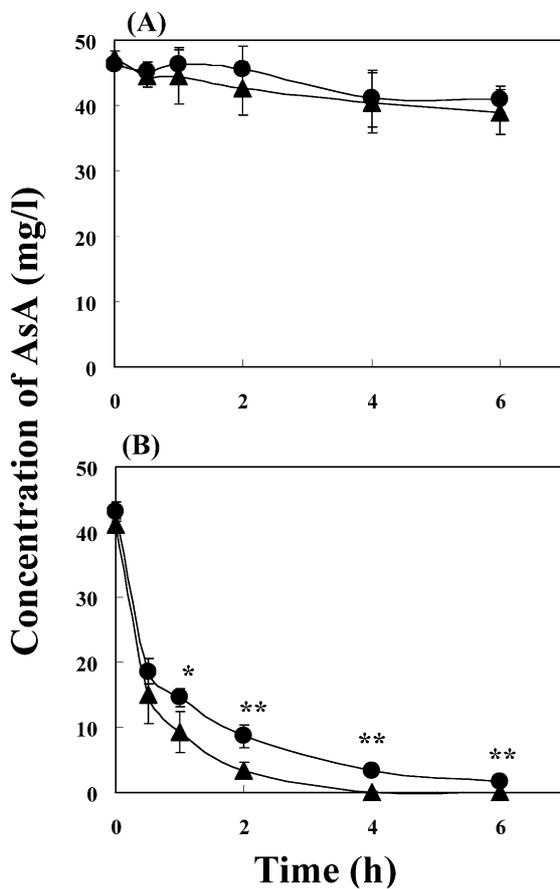


Fig. 4. Remaining amounts of AsA under *quasi*-physiological conditions at 37°C.

Each sample solution containing 50 mg/l of AsA was incubated in simulated gastric juice (pH 1.8 (A)) or simulated intestinal juice (pH 8.5 (B)) for the indicated time at 37°C. The remaining amounts of AsA were measured by the dinitrophenylhydrazine-HPLC method. Each value is the mean \pm standard deviation of five different replicates. ▲: AsA alone, ●: premixed GSE with AsA. *: $p < 0.05$, **: $p < 0.01$ vs. AsA alone.

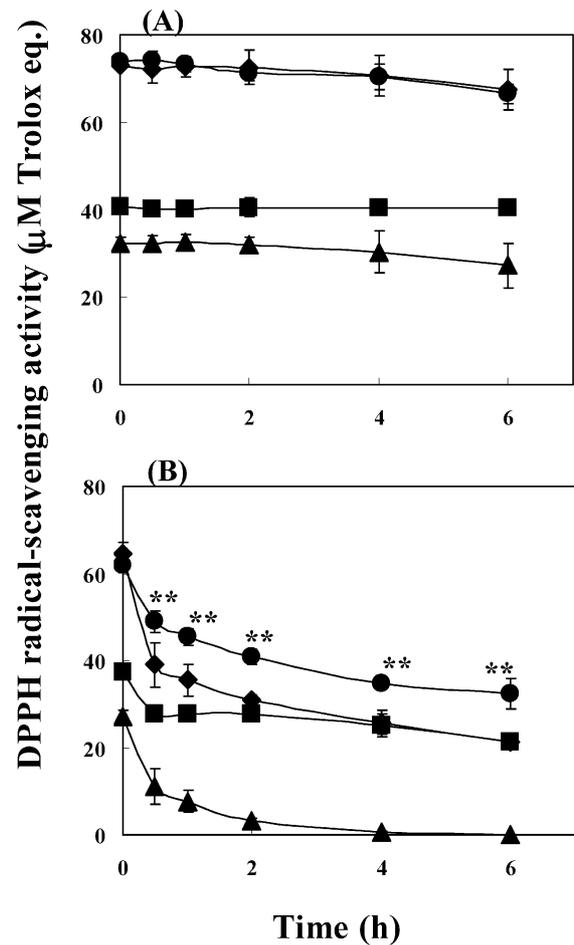


Fig. 5. Remaining DPPH radical-scavenging activity under *quasi*-physiological conditions at 37°C.

Each sample solution (50 mg/l) was incubated in simulated gastric juice (pH 1.8 (A)) or simulated intestinal juice (pH 8.5 (B)) for the indicated time at 37°C, and the remaining DPPH radical-scavenging activity was measured by the colorimetric method. Each value is the mean \pm standard deviation of five different replicates. ■: GSE alone, ▲: AsA alone, ◆: GSE plus AsA, ●: premixed GSE with AsA. **: $p < 0.01$ vs. GSE plus AsA.

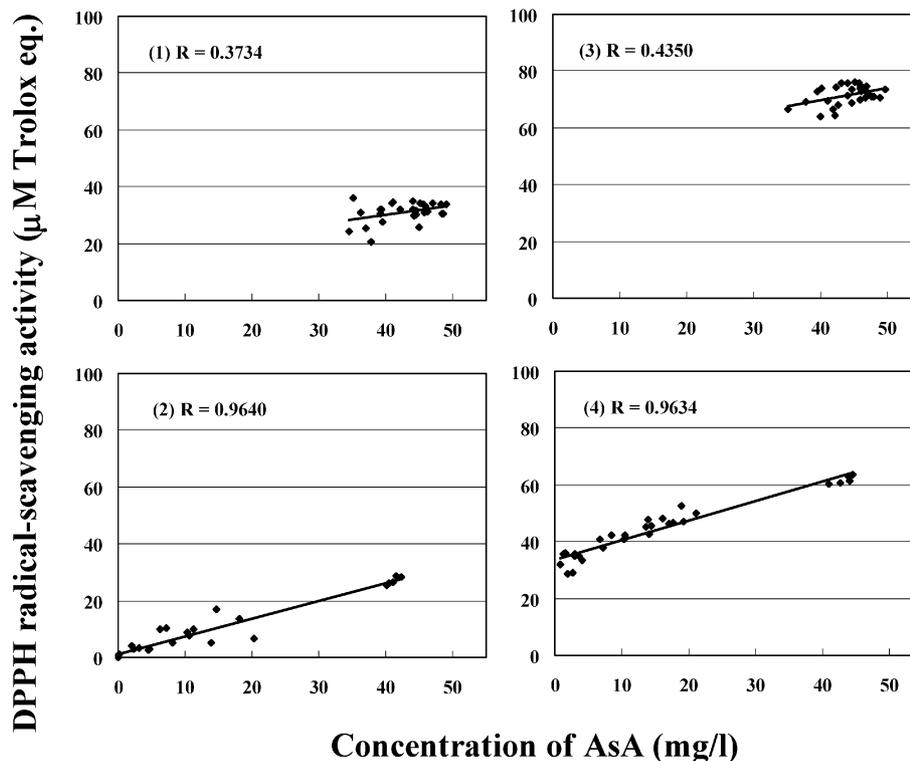


Fig. 6. Correlation between the remaining amounts of AsA and DPPH radical-scavenging activity under *quasi*-physiological conditions.

(1): AsA alone in simulated gastric juice (pH 1.8), (2): AsA alone in simulated intestinal juice (pH 8.5), (3): premixed GSE with AsA in simulated gastric juice (pH 1.8), (4): premixed GSE with AsA in simulated intestinal juice (pH 8.5).

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