Antioxidant Activities of Dioscorin, the Storage Protein of Yam (*Dioscorea batatas* Decne) Tuber

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Dioscorin, the storage protein of yam (*Dioscorea batatas* Decne) tuber (which is different from dioscorine found in tubers of *Dioscorea hirsuta*), was purified to homogeneity after DE-52 ion exchange column according to the methods of Hou et al. (*J. Agric. Food Chem.* **1999**, *47*, 2168−2172). A single band of 32 kDa dioscorin was obtained on a sodium dodecyl sulfate−polyacrylamide gel electrophoresis gel with 2-mercaptoethanol treatment. This purified dioscorin was shown by spectrophotometric method to have scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in a pH-dependent manner. There is a positive correlation between scavenging effects against DPPH (8−46%) and amounts of 32 kDa dioscorin (5.97−47.80 nmol) added in Tris-HCl buffer (pH 7.9), which are comparable to those of glutathione at the same concentrations. Using electron paramagnetic resonance (EPR) spectrometry for DPPH radical detection, it was found that the intensities of the EPR signal were decreased by 28.6 and 57 nmol of 32 kDa dioscorin in Tris-HCl buffer (pH 7.9) more than in distilled water compared to controls. EPR spectrometry was also used for hydroxyl radical detection. It was found that 32 kDa dioscorin could capture hydroxyl radical, and the intensities of the EPR signal were significantly decreased dose-dependently by 1.79−14.32 nmol of 32 kDa dioscorin ($r = 0.975$) compared to the control. It is suggested that 32 kDa dioscorin, the storage protein of yam tuber, may play a role as antioxidant in tubers and may be beneficial for health when people take it as a food additive or consume yam tubers.

Keywords: *Antioxidant; dioscorin; 1,1-diphenyl-2-picrylhydrazyl (DPPH); electron paramagnetic resonance (EPR); hydroxyl radical; yam*

INTRODUCTION

Active oxygen species (or reactive oxygen species) and free radical-mediated reactions are involved in degenerative or pathological processes such as aging (*1*, *2*), cancer, coronary heart disease, and Alzheimer's disease (*3*−*6*). There are many epidemiological results revealing an association between people who have a diet rich in fresh fruits and vegetables and a decrease in the risk of cardiovascular diseases and certain forms of cancer (*7*). Several authors have studied the natural compounds in fruits and vegetables for their antioxidant activities, such as echinacoside in *Echinaceae* root (*8*), anthocyanin (*9*), phenolic compounds (*10*), water extracts of roasted *Cassia tora* (*11*), and whey proteins (*12*−*14*).

Dioscorin, the storage protein of yam tuber (which is different from dioscorine found in *Dioscorea hirsuta*), accounted for ∼90% of extractable water-soluble proteins from different yam species (*Dioscorea batatas*, *D. alata*, and *D. pseudojaponica*) as estimated by an immunostaining method (*15*), and all of them exhibited carbonic anhydrase and trypsin inhibitor (TI) activities (*15*, *16*). We also proved that dioscorin exhibited both dehydroascorbate reductase and monodehydroascorbate reductase activities and might respond to environmental stresses (*17*), a similar situation to trypsin inhibitor, the storage protein of sweet potato roots (*18*). In our recent paper, the 33 kDa trypsin inhibitor, one root storage protein of sweet potato, exhibited antioxidant activities against different radicals (*19*). In this work we report for the first time that 32 kDa dioscorin, the major yam tuber storage protein, had scavenging activities against both 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals analyzed by a spectrophotometric method or electron paramagnetic resonance (EPR) spectrometry.

MATERIALS AND METHODS

Dioscorin Purified from Yam Tuber. Fresh yam (*D. batatas* Decne) tubers were imported from Japan via a wholesaler immediately for dioscorin extraction. After washing and peeling, the tubers were cut into strips for dioscorin extraction and purification according to the method of Hou et al. (*16*). After extraction and centrifugation, the crude extracts of yam tubers were loaded directly onto a DE-52 ion exchange column. After washing with 3 column volumes of 50 mM Tris-HCl buffer (pH 8.3), the adsorbed dioscorins were eluted batchwise with the same washing buffer containing 150 mM NaCl. The eluted fraction was collected and concentrated with Ultrafree 4 (molecular weight cutoff is 5 kDa, Millipore Co.,

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Bedford, MA). The concentrated dioscorin solution was dialyzed against deionized water overnight and lyophilized for further use.

Protein Staining on SDS-PAGE Gels. Four parts of samples were mixed with one part of sample buffer, namely, 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without 2-mercaptoethanol and heated at boiling water temperature for 5 min, cooled to ambient temperature, and electrophoresed according to the method of Laemmli (*20*). Coomassie brilliant blue R-250 was used for protein staining (*21*).

Scavenging Activity of 32 kDa Dioscorin or Glutathione against DPPH Radical Determined by Spectrophotometry. The scavenging activity of dioscorin or glutathione against DPPH radical was measured according to the method of Hou et al. (*19*) or Yamaguchi et al. (*22*) with some modifications. The 1.2 mL sample solution containing different amounts of 32 kDa dioscorin (5.97, 11.95, 23.89, and 47.79 nmol) or glutathione (6.6, 13.2, 26.4, and 66 nmol) was added to 0.1 mL of 1 M Tris-HCl buffer (pH 7.9) and then mixed with 1.2 mL of 80 μM DPPH in methanol for 20 min under light protection at room temperature. The absorbance at 517 nm was determined. Deionized water was used instead of sample solution for control experiments. The decrease of absorbance at 517 nm was calculated and expressed as ΔA_{517nm} for scavenging activity.

Effects of pH on the Scavenging Activity of 32 kDa Dioscorin against DPPH Radical by Spectrophotometry. The 1.2 mL sample of 5.97 nmol of 32 kDa dioscorin was added to 0.1 mL of different pH values of 1 M phosphate buffer (6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) or 1 M Tris-HCl buffer (pH 7.0, 7.5, 7.9, 8.0, and 8.5) and then mixed with 1.2 mL of 80 μM DPPH in methanol for 20 min under light protection at room temperature. The absorbance at 517 nm was determined. Each phosphate buffer or Tris-HCl buffer of different pH value was used instead of sample solution for corresponding control experiments. The ΔA_{517nm} of dioscorin at 1 M Tris-HCl buffer (pH 7.9) was assumed as 100% and expressed as relative scavenging activity against DPPH radical.

Scavenging Activity of 32 kDa Dioscorin against DPPH Radical Determined by EPR Spectrometry. The sample solution contained 25 μL of two different amounts of 32 kDa dioscorin (800 and 1600 μg corresponding to 28.6 and 57 nmol, respectively) in deionized water. Then, 10 μL of deionized water or 1 M Tris-HCl buffer (pH 7.9) was added to the sample solution. The mixture was transferred to the EPR quartz cell, and then deionized water was added to 250 μL and placed at the cavity of the EPR spectrometer; finally, 250 μL of DPPH in methanol (2 mM) was added. The relative intensity of the DPPH spin signal was measured. The deionized water was used instead of sample solution for control experiments. EPR spectra were recorded with a modulation frequency 100 kHz and at 9.75 GHz resonant frequency, and the instrumental settings were as follows: microwave power, 2.007e-001 mW; center field, 3476 ± 50 G; modulation amplitude, 3.0 G; conversion time, 82 ms; time constant, 655 ms; sweep time, 84 s.

Scavenging Activity of 32 kDa Dioscorin against Hydroxyl Radical Determined by EPR Spectrometry. The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*23*). The mixture included different amounts of 32 kDa dioscorin (50, 100, 200, and 400 μg corresponding to 1.79, 3.58, 7.16, and 14.32 nmol, respectively), 5 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an EPR quartz cell and placed at the cavity of the EPR spectrometer, and then hydrogen peroxide was added to a final concentration of 0.25 mM in 500 μL of total volumes. Deionized water was used instead of sample solution for control experiments. After 40 s, the relative intensity of the signal of the DMPO−OH spin adduct was measured. All EPR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 EPR spectrometer equipped with WIN-EPR SimFonia software version 1.2. The conditions of EPR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min.

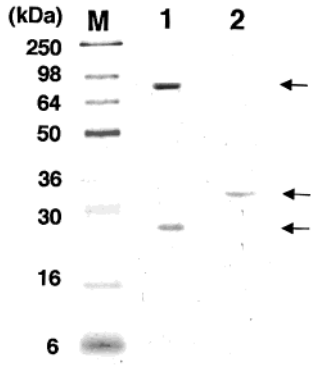

Figure 1. Protein stainings of dioscorin without (lane 1) and with (lane 2) 2-mercaptoethanol treatment on an SDS-PAGE gel after DE-52 ion exchange column purification. M indicated the Seeblue prestained markers of SDS-PAGE; 2 μg of protein was loaded in each well.

Materials. Tris and electrophoretic reagents were purchased from E. Merck Inc. (Darmstadt, Germany); Seeblue prestained markers for SDS-PAGE were from Novex (San Diego, CA); DE-52 anion exchange resin was from Whatman Inc.; DPPH, Coomassie brilliant blue G-250, DMPO, and ferrous sulfate were purchased from Sigma Chemical Co. (St. Louis, MO); hydrogen peroxide (33%) was from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals and reagents were from Sigma Chemical Co.

RESULTS AND DISCUSSION

Dioscorin Purified from Yam Tuber. Dioscorin was purified from yam (*D. batatas* Decne) tubers according to the method of Hou et al. (*16*). The purity of dioscorin was determined by SDS-PAGE gels. Figure 1 shows the protein stainings of dioscorin without (lane 1) and with (lane 2) 2-mercaptoethanol treatment. Two protein bands with molecular masses of 28 and 82 kDa were found without 2-mercaptoethanol treatment (lane 1), and a single band (lane 2) with molecular mass of 32 kDa was found after 2-mercaptoethanol treatment. These results were the same as those of Hou et al. (*16*). All bands cross-reacted with a polyclonal antibody raised against dioscorin with a molecular mass of 28 kDa (*15*). This purified dioscorin was lyophilized for further investigations.

Scavenging Activity of 32 kDa Dioscorin or Glutathione against DPPH Radical Determined by Spectrophotometry. The DPPH radical has been widely used in model systems to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude mixtures such as methanol extracts of plants (*8*, *9*, *24*, *25*). However, few studies have reported on proteins, except antioxidative enzymes, with regard to the subject of direct antiradical effects. Therefore, we used 32 kDa dioscorin to test the scavenging activities against the DPPH radical (Figure 2). Figure 2 shows that the scavenging activity of 32 kDa dioscorin against the DPPH radical is concentration-dependent (Figure 2, lower panel). This is the first report that 32 kDa dioscorin, the major storage protein of yam tubers, could capture the DPPH radical. Absorbance decreases (A_{517nm}) as a result of a color change from purple to yellow as the DPPH radical is scavenged by antioxidants through donation of hydrogen to form the reduced DPPH−H (*9*,

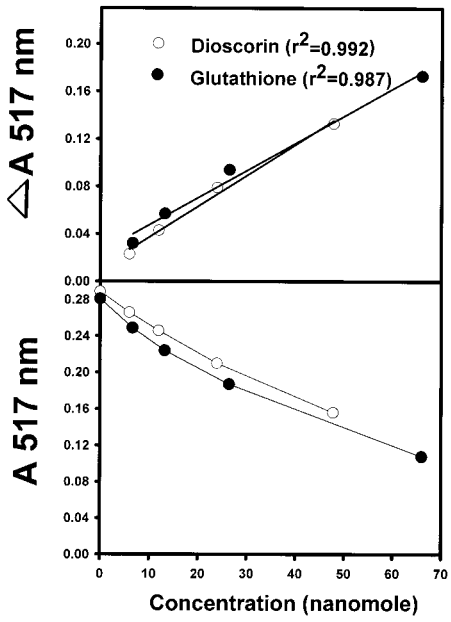

Figure 2. Scavenging activity of 32 kDa yam dioscorin (5.79, 11.95, 23.89, and 47.79 nmol) or glutathione (6.6, 13.2, 26.4, and 66 nmol) against DPPH radicals in Tris-HCl buffer (pH 7.9) determined by spectrophotometric method (lower panel). Each linear regression was plotted with data pairs of scavenging activity (ΔA_{517nm}) and concentration of 32 kDa dioscorin or glutathione (upper panel).

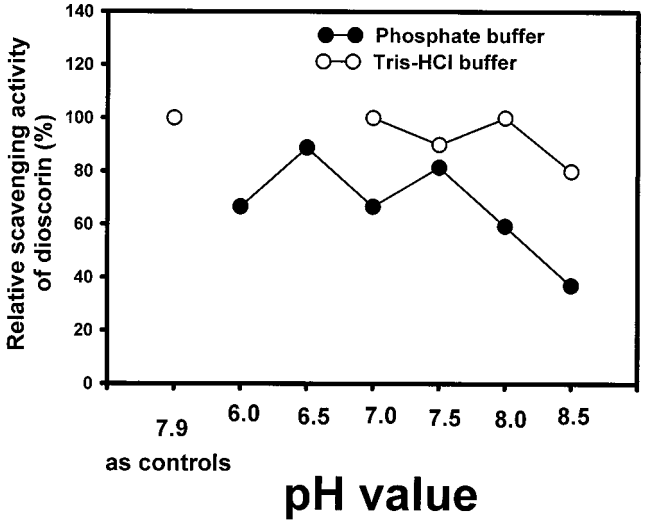

Figure 3. Effects of pH on the scavenging activity of 32 kDa dioscorin (5.97 nmol) against DPPH radical determined by spectrophotometry. The phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) or Tris-HCl buffer (pH 7.0, 7.5, 7.9, 8.0, and 8.5) was used. The absorbance at 517 nm was determined. Phosphate buffers of different pH values and Tris-HCl buffers were used instead of sample solution for corresponding control experiments. Relative scavenging activities of dioscorin against DPPH radical (ΔA_{517nm}) were shown with that of dioscorin in 1 M Tris-HCl buffer (pH 7.9) as an arbitrary standard (100%).

26). There was a positive correlation between scavenging effects against DPPH (8−46%) and amounts of 32 kDa dioscorin (5.97−47.80 nmol) added in Tris-HCl buffer (pH 7.9), which is comparable to those of glutathione at the same concentrations (Figure 2, upper panel). In our recent paper, the 33 kDa trypsin inhibitor, one storage protein of sweet potato roots, exhibited antioxidant activities against DPPH radicals. The scavenging effects of the 33 kDa trypsin inhibitor were about one-third that of glutathione as a scavenger of DPPH (*19*). The dioscorin had a higher scavenging ability than that of sweet potato 33 kDa trypsin inhibitor against DPPH radicals. Allen and Wrieden (*12*, *13*) found that whey proteins (α-lactalbumin and *β*-lactoglobulin) exhibited antioxidative activities against copper-catalyzed lipid oxidation. Tong et al. (*14*) also found that whey protein fractions with molecular mass >3.5 kDa exhibited antioxidative activities against lipid peroxidation and peroxyl radical. They pointed out that free sulfhydryl groups in whey proteins might mainly contribute the antioxidative activities. We found that yam dioscorins (*17*) and sweet potato trypsin inhibitors (*18*) exhibited dehydroascorbate reductase activities which were independent of glutathione, and intermolecular thiol−disulfide interchanges of TIs or dioscorins were found during dehydroascorbate reduction. It was postulated that the free sulfhydryl groups in sweet potato TIs or in yam dioscorin could reduce dehydroascorbate to regenerate ascorbate to prevent oxidative damage to sweet potato roots or yam tubers. The scavenging activity of 32 kDa dioscorin against the DPPH radical might be largely due to its free sulfhydryl groups.

Effects of pH on the Scavenging Activity of 32 kDa Dioscorin against the DPPH Radical Determined by Spectrophotometry. Phosphate buffers (pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) and Tris-HCl buffers (pH 7.0, 7.5, 7.9, 8.0, and 8.5) were used to investigate the pH effects on dioscorin (5.97 nmol) scavenging activities against DPPH radicals. Relative scavenging activities of dioscorin against DPPH radical (ΔA_{517nm}) are shown in Figure 3 with that of dioscorin in 1 M Tris-HCl buffer (pH 7.9) as an arbitrary standard (100%). From Figure 3, it was found that the scavenging activities of dioscorin against DPPH radicals were pH-dependent and the scavenging activity was higher in Tris-HCl buffer than in phosphate buffer at the same pH value. The highest scavenging activity was found in Tris-HCl buffer of pH 8.0, which was the same as that of pH 7.9. It was also found that the absorbance at 517 nm of DPPH in control experiments increased as pH increased. For example, 0.276 (pH 6.0), 0.286 (pH 6.5), 0.293 (pH 7.0), 0.306 (pH 7.5), 0.313 (pH 8.0), and 0.317 (pH 8.5) were found in phosphate buffer; however, 0.281 (pH 7.9) was obtained in Tris-HCl buffer. At the same pH value, DPPH in phosphate buffer had a much higher absorbance at 517 nm than it did in Tris-HCl buffer. These results revealed that the different buffers might affect the hydrogen abstraction from dioscorin for scavenging activity against the DPPH radical and will need further investigations.

Scavenging Activity of 32 kDa Dioscorin against the DPPH Radical Determined by EPR Spectrometry. Besides the spectrophotometric method, EPR spectrometry was also used to evaluate the scavenging activity of 32 kDa yam dioscorin against DPPH radicals. Figure 4 shows the scavenging activity against DPPH radicals of 32 kDa dioscorin at two different levels [28.6 nmol (Figure 4B,E) and 57 nmol (Figure 4C,F)] in deionized water (Figure 4B,C) or in pH 7.9 Tris-HCl buffer (Figure 4E,F) (Figure 4A,D as controls, respectively). It was found that 32 kDa dioscorin could capture DPPH radicals in deionized water (Figure 4B,C) or in Tris-HCl buffer (Figure 4E,F) as determined by EPR spectrometric method. Although the intensities of DPPH signals in deionized water (Figure 4A) were much greater than those in Tris-HCl buffer (pH 7.9) (Figure 4D) under the same experimental conditions, the in-

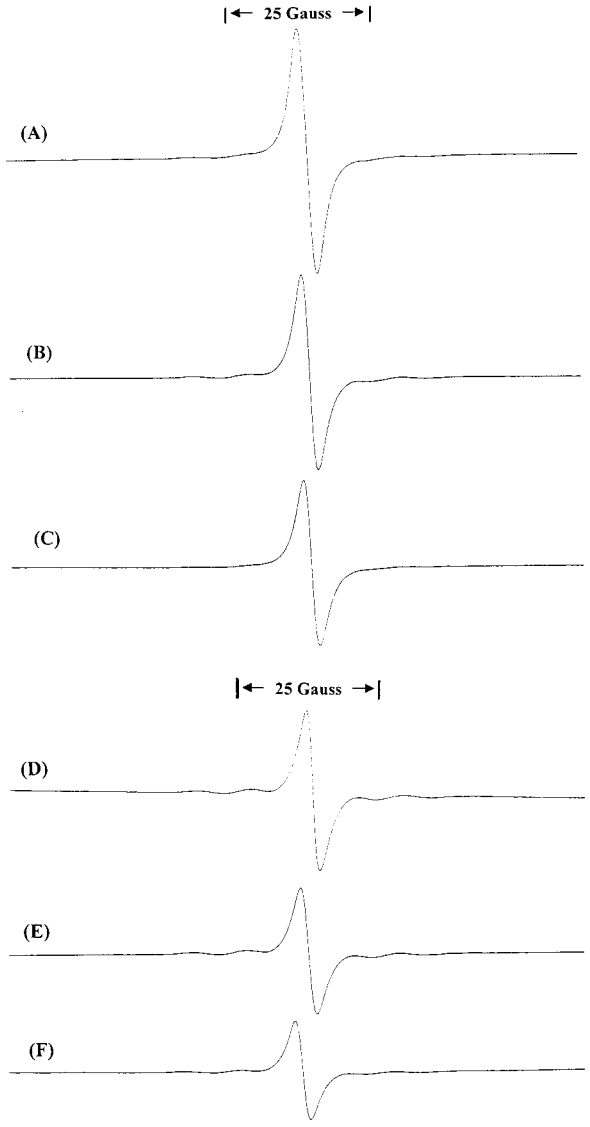

Figure 4. Scavenging activity of 32 kDa dioscorin against DPPH radical determined by EPR spectrometry. Sample solutions contained two different amounts of 32 kDa dioscorin [28.6 nmol (B, E) and 57 nmol (C, F)] in deionized water. The deionized water was used instead of sample solution for control experiments (A, D). Then, 10 μL of deionized water (A−C) or 10 μL of 1 M Tris-HCl buffer (pH 7.9) (D−F) was added to the sample and control solutions, and finally 250 μL of DPPH in methanol (2 mM) was added; the intensities of DPPH signals were then determined.

tensities of the EPR signal were decreased in Tris-HCl buffer (pH 7.9) (Figure 4E,F) more significantly than in distilled water (Figure 4B,C) by 28.6 and 57 nmol of 32 kDa dioscorin compared to controls. From the height of DPPH signal intensities, there were about 19.44 and 31.67% reductions, respectively, in deionized water by 28.6 nmol (Figure 4B) and 57 nmol (Figure 4C) of dioscorin compared to the control (Figure 4A). On the other hand, there were about 24.58 and 41.34% reductions, respectively, in Tris-HCl buffer (pH 7.9) by 28.6 nmol (Figure 4E) and 57 nmol (Figure 4F) of dioscorin compared to the control (Figure 4D).

Scavenging Activity of 32 kDa Dioscorin against Hydroxyl Radical Determined by EPR Spectrometry. The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*23*) and was trapped by DMPO to form the DMPO−OH adduct. The intensities of the DMPO−OH spin signal

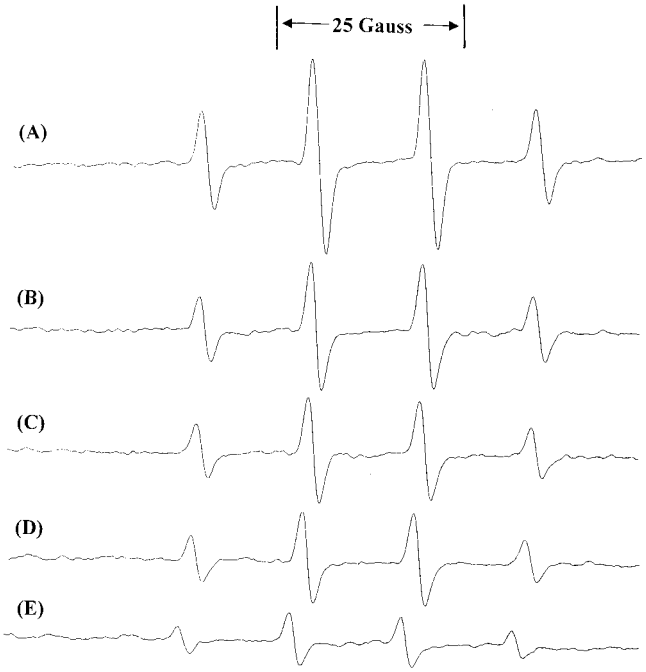

Figure 5. Scavenging activity against the hydroxyl radical by 32 kDa yam dioscorin measured by EPR spectrometry: (A) controls; (B) 50 μg (1.79 nmol), (C) 100 μg (3.58 nmol), (D) 200 μg (7.16 nmol), and (E) 400 μg (14.32 nmol) of 32 kDa dioscorin were added.

in EPR spectrometry were used to evaluate the scavenging activity of 32 kDa yam dioscorin against hydroxyl radical. Figure 5 shows the scavenging activity against the hydroxyl radical with different amounts of 32 kDa dioscorin [(A) controls; (B) 50 μg (1.79 nmol), (C) 100 μg (3.58 nmol), (D) 200 μg (7.16 nmol), and (E) 400 μg (14.32 nmol)] monitored by EPR spectrometry. The effect of 32 kDa dioscorin as a scavenger of hydroxyl radical was evident as decreased intensities of DMPO−OH signals were observed. EPR signals were significantly decreased by 1.79−14.32 nmol of 32 kDa dioscorin compared to the control with a positive correlation between two parameters ($r = 0.975$). On the basis of DMPO−OH signal intensities, there were about 33, 47, 52, and 72% reductions, respectively, by 1.79, 3,58, 7.16, and 14.32 nmol of dioscorin compared to controls (Figure 5B−E). Figure 5 provides the first piece of evidence that yam dioscorin exhibited scavenging activity against the hydroxyl radical as shown by EPR spectrometry. These results were similar to those of Hou et al. (*19*).

In conclusion, both DPPH and hydroxyl radicals were used as model systems to evaluate the antioxidant ability of dioscorin, the storage protein of yam tubers. Our results reveal that 32 kDa dioscorin could capture both radicals in a concentration-dependent manner and may play a role as an antioxidant in tubers.

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