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Isolation and Characterization of Antioxidant Protein Fractions from Melinjo (*Gnetum gnemon*) Seeds

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ABSTRACT: The protein from the seeds of melinjo (*Gnetum gnemon*) was purified using a precipitation method and ion exchange chromatographic techniques to identify the potent antioxidant and free radical scavenging activities. Two antioxidant protein fractions were isolated from *G. gnemon* seed with molecular weights of approximately 30 kDa (Gg-AOPI) and 12 kDa (Gg-AOPII) by SDS-PAGE. The N-terminal amino acid sequence of Gg-AOPII is Gly-Asn-Gly-Lys-Ala-Thr-Val-Ala-Ile-Leu-Val-Lys-Glu-Lys-Val-Glu-Tyr-Gly-Glu-Glu, and the result of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis showed that they were distinct from each other; no protein in database matching was found to both Gg-AOPI and Gg-AOPII. The antioxidant or free radical scavenging activities of Gg-AOPs were investigated by employing in vitro assay systems including the inhibition of linoleic acid autoxidation, scavenging effect on α,α-diphenyl-β-picrylhydrazyl free radical (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), reducing power, chelating abilities of metal ions Cu^{2+} and Fe^{2+}, and protections against hydroxyl radical-mediated DNA damages. The result showed that two protein fractions exhibited significant ($p < 0.05$) antioxidant activities against free radicals such as DPPH, ABTS, and superoxide anion and showed activities similar to those of glutathione (G-SH) and BHT in a linoleic acid emulsion assay system. Moreover, Gg-AOPI and Gg-AOPII also exhibited notable reducing power and strong chelating effect on Fe^{2+} and protected hydroxyl radical induced oxidative DNA damage. The data obtained by the in vitro systems obviously established the antioxidant potency of Gg-AOPs.

KEYWORDS: antioxidant, free radical scavenging, protein fractions, *Gnetum gnemon*

INTRODUCTION

Reactive oxygen species (ROS) have effects on many substances in the human body, such as fatty acids, proteins, and DNA.[1] Generation of ROS or free radicals such as superoxide, hydroxyl radical, and hydrogen peroxide during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress.[2,3] Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer, and the aging process.[4−10] All organisms have antioxidant systems that are able to control and counter the onslaught of free radical mediated oxidative damage. Therefore, dietary sources have been recognized as safer and effective antioxidants in the context of their efficiency and nontoxicity. The intake of fruits and vegetables containing high amounts of antioxidative nutraceuticals has been associated with the balance of the free radical or antioxidant status, which helps to minimize the oxidative stress in the body and to reduce the risks of diseases.

Recently, the use of natural products, such as protein extracts or purified proteins, as antioxidants has attracted particular interest. Many food proteins, including milk proteins, such as lactoferrin, β-lactoglobulin, and casein, soy proteins, canola proteins, egg albumen proteins and egg yolk phosvitin, maize zein, potato patatin, yam dioscorin, chickpeas and white beans, and *Ginkgo biloba* seeds, were reported to have antioxidant activity.[11−19]

As an archaic living fossil, *Gnetum gnemon* L. (Gnataceae) is one of the oldest species of tree, with great antiadversity ability, which has existed on the earth for 200 million years.[20] The gnemon tree, *G. gnemon* L. (Gnataceae), is cultivated in Indonesia, Malaysia, and other southeast Asian islands for its seeds and is used as food in Indonesia. *G. gnemon* (melinjo) seeds contain a high concentration of protein by 9−11% of the seed.[21] These seeds may be considered as a suitable source of functional protein and nutraceutical food supplement with high bioavailability. However, there is no study on the use of the *G. gnemon* seed protein as a source of free radical scavenger or antioxidant protein for nutraceutical food supplement.

In this study, a systematic search was conducted to isolate and characterize the two active antioxidant protein fractions (Gg-AOPI and Gg-AOPII) from *G. gnemon* seed. The antioxidant properties of the two protein fractions were compared in antioxidant and free radical scavenging activities.

MATERIALS AND METHODS

Extraction Methods. *G. gnemon* seeds were collected during June−July 2008 from the plants from the collection of the Faculty of Agriculture, University of Jember, South East of Java, Indonesia. After washing and peeling, 25 g of sample seed was ground in a stainless steel

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blender (model 38BL19/CB10), and the resulting meal was extracted with 50 mL of an ice-cold extraction of 10 mM phosphate buffer (pH 6.5) for 2−4 h at 4 °C. The resulting homogenate was run through a filter cloth to remove larger particulate materials and then clarified by centrifugation (10000 rpm for 15 min) in a large-capacity centrifuge. Solid ammonium sulfate was added to the supernatant to obtain 0−30% relative saturation and the precipitate allowed to form overnight with stirring at 4 °C. Following centrifugation at 10000 rpm for 15 min, the precipitated protein was taken and resuspended in a minimal volume of extraction buffer and dialyzed in nitrocellulose membrane. This fraction was further purified using combination column chromatography.

Purification of Antioxidant Protein from *G. gnemon* (Gg-AOP). Ammonium-precipitated fraction (48 mL) was loaded onto a CM-cellulose column (2.5 × 75 cm), equilibrated with 0.3 M ammonium acetate buffer (pH 5.2), and washed with 2 L of the same buffer. A 2000 mL gradient of 0.4−1.2 M ammonium acetate buffer (pH 5.2) was applied at a flow rate of 2 mL min^{-1}. Each fraction (2 mL) was collected, and fractions representing absorbance peaks at 280 nm were pooled, resuspended in 0.01% acetic acid, lyophilized again, and stored at −20 °C. The active fraction was applied onto DEAE-cellulose column (2.5 × 75 cm). A linear gradient of 0−1 M NaCl in 10 mM Tris-HCl buffer (pH 6.8) was maintained at a flow rate of 1 mL min^{-1}. Fractions collected from the column were assayed for their antioxidant activity against DPPH, as detailed below. The protein content of the fractions was also monitored by measuring absorbance values at 280 nm. Being determined for the profiles of antioxidant activity and protein, the most active fractions of separated proteins were combined and once more tested for antioxidant activity. The antioxidant activity in the purification table was expressed as vitamin C equivalents antioxidant capacity (VCEAC; mg vit-C).[22] The specific antioxidant activity was calculated by dividing the total antioxidant activity by protein content, determined according to the Bradford method. The obtained protein fractions were then dialyzed for 24 h (against 3−2 L of distilled water) at 4 °C, lyophilized, and stored at −18 °C.

Determination of Molecular Weight and Amino Acid Composition. SDS-PAGE electrophoresis was performed in accordance with the method of Laemmli.[23] As the gel, use was made of a 15% and the protein was detected with the use of CBB. To roughly estimate the molecular weight and amount of the protein, molecular weight markers (LMW, manufactured by Pharmacia LKB Co. Ltd.; 116, 97, 66, 30, 21, 14, and 6 kDa, descending from larger to smaller size) were electrophoresed in such a manner that a single band stood for 20 ng.

The amino acid composition of antioxidant protein fractions was analyzed according to the method of Bidlingmeyer et al.[24] The hydrolysis of the antioxidant protein fraction preparation (1 mg) was carried out in evacuated, sealed, thick-walled borosilicate glass tubes for 24 h at 110 °C with 6 M HCl, and the hydrolysate was subjected to analysis with a JLC-500/V Amino Tac Amino Acid Analyzer (JEOL Ltd., Tokyo, Japan).

MALDI-TOF-MS. Protein digestion and all procedures for MALDI-TOF-MS (Voyager STR, PerSeptive Biosystems, Framingham, MA) analysis and searching were followed as described.[25] The sample was first separated by 12.5% SDS-PAGE. Bands containing the proteins of interest were cut into small pieces, washed with 50% acetonitrile in 0.1 M NH_4HCO_3, and vacuum-dried. The gel pieces were reduced for 45 min at 55 °C in 10 mM DTT in 0.1 M NH_4HCO_3. After that, the DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and incubated for 30 min at room temperature in the dark. Gel pieces were washed with 50% acetonitrile in 0.1 M NH_4HCO_3 and dried in a SpeedVac evaporator. The dried gel pieces were swollen in a minimum volume of 10 μL of digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/μL trypsin (Promega, sequencing grade) and incubated at 37 °C overnight. The digestion mixture was redissolved using a solution of distilled water/acetonitrile/trifluoroacetic acid (93:5:2). The samples were sonicated for 5 min and centrifuged for 2 min. The matrix solution [dissolved α-cyano-4-hydroxycinnamic acid (Sigma) in acetone (40 mg/mL) and nitrocellulose in acetone (20 mg/mL)], the nitrocellulose solution, and isopropanol were mixed 100:50:50. The samples were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer with the following parameters. Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76%, a guide wire voltage of 0.010%, and a delay time of 150 ns. For data processing, MoverZ (http://bioinformatics.genomicsolutions.com) software was used. The acquired peak lists were analyzed by database (National Center for Biotechnology Information nonredundant, NCBInr) searches for identification by peptide mass fingerprint (PMF) using MASCOT (www.matrixscience.com). The searching parameters were followed by which other green plants was chosen for the taxonomic category. The results with MOWSE scores of >65 ($p < 0.05$) were considered to be valuable.

N-Terminal Amino Acid Sequencing. The amino acid sequence of the N terminus was determined on a protein sequencer (ABI Procise 491 HT protein sequencer, Applied Biosystems) using automated Edman degradation. After SDS-PAGE, purified protein was transferred to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie Brilliant Blue R250. After the Gg-AOP was eluted from the membrane, the sequence of the amino acid residues at the N terminus was determined, which was compared with known sequences by using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

Scavenging Effect on DPPH Free Radical. The scavenging effect of Gg-AOP fractions on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was measured according to the method of Shimada et al.[26] with slight modifications. A volume of 1 mL of each sample was added to 2 mL of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. The antioxidant activity was calculated as $[(A_c - A_s)/A_c] \times 100\%$, where A_c and A_s are the absorbance of the control and sample, respectively.

ABTS Radical Cation (ABTS) Scavenging Activity Assay. ABTS radical scavenging activities of Gg-AOP fractions were determined as described by You et al.[27] with slight modification. The ABTS solution was prepared with final concentration of 7 mM ABTS and 2.45 mM potassium persulfate. The mixture was left in the dark at room temperature for 12−16 h before use. Prior to the assay, the ABTS solution was diluted with 0.2 M sodium phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. Then 40 μL of the sample containing 2.0 mg/mL was added to 4 mL of diluted ABTS solution. The mixture was shaken vigorously for 30 s and left in the dark for 6 min. An equivalent volume of distilled water instead of the sample was used for the blank. The absorbance of the resultant solution was measured at 734 nm. For comparison, the ABTS scavenging activity of glutathione (G-SH) was also tested. The ABTS scavenging activity was calculated as $[(A_c - A_s)/A_c] \times 100\%$, where A_c and A_s are the absorbance of the control and sample, respectively.

Superoxide Anion Radical scavenging activity. For the superoxide anion scavenging activity measurement, the autoxidation of a pyrogallol method described by Tang et al.[28] was followed with slight modification. Briefly, 0.1 mL of Gg-AOP fraction was mixed with 1.8 mL of 50 mM Tris-HCl buffer (pH 8.2). The mixture was incubated at 25 °C for 10 min, and then 0.1 mL of 10 mM pyrogallol (dissolved in 10 mM HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for sample was calculated as the slope of the absorbance line (ΔA_s). The autoxidation rate pyrogallol for control was measured with 1.0 mL of double-distilled water (ΔA_0). For comparison, the superoxide anion scavenging activity of G-SH was also tested. The superoxide anion scavenging activity was calculated as $[(\Delta A_0 - \Delta A_s)/\Delta A_0] \times 100\%$.

Inhibition of Linoleic Acid Oxidation. The antioxidant activity of Gg-AOP fraction with different periods of incubation was measured in a linoleic acid model system according to the methods of Osawa and Namiki[29] with some modifications. Each sample (10 mg) was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and added to a solution of 0.15 mL of linoleic acid and 10 mL of 99.5% ethanol. Then, the total volume was adjusted to 25 mL with distilled water. The mixture was incubated in a conical flask with a screw cap at 40 °C in a dark room. The degree of oxidation was evaluated by measuring the ferric thiocyanate values according to the method of Mitsuda et al.[30] The reaction solution (100 mL) incubated in the linoleic acid model system described herein was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 0.02 M ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm, following color development for the complex of $FeCl_2$ and thiocyanate, at different intervals during the incubation period at 40 °C.

Reducing Power Assay. The reducing power of protein isolated was determined according to the method of Ahmadi et al.[31] with slight modification. The Gg-AOP fractions were dissolved in distilled water to obtain a concentration of 2.0 mg/mL. An aliquot (2.0 mL) was mixed with 2.0 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.0 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 2.0 mL of 10% trichloroacetic acid was added to the mixture. After centrifugation at 3000 rpm for 10 min, 2.0 mL of the supernatant was collected and mixed with 2.0 mL of distilled water and 4.0 mL of 0.1% (w/v) $FeCl_3$. After the mixture had stood at room temperature for 10 min, the absorbance was measured at 700 nm. An equivalent volume of distilled water instead of the sample was used as the blank. The reducing power was expressed as equivalents of vitamin C (mg vit-C).[32]

Chelating of Metal Ions Cu^{2+} and Fe^{2+}. The Cu^{2+}-chelating ability was measured according to the method of Zhu et al.,[33] with minor modification. An aliquot (1 mL) of Gg-AOP fraction solution was mixed with 1 mL of pyridine (10%, pH 7.0) and 20 μL of 0.1% pyrocatechol violet. After the addition of 1 mL of protein isolated (Gg-AOPI and Gg-AOPII), the disappearance of blue color, due to dissociation of Cu^{2+}, was recorded by measuring the absorbance at 632 nm after 5 min of reaction. An equivalent volume of distilled water instead of the sample was used as blank (A_0). The chelating of Fe^{2+} by the sample was estimated according to the method of Dinis et al.[34] The Gg-AOP fraction (1 mL) was added to a solution of 2 mM ferrous chloride (0.1 mL) and 3.7 mL of deionized water. After 3 min, the reaction was inhibited by the addition of 5 mM ferrozine (0.2 mL). The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the resulting solution was measured at 562 nm. An equivalent volume of distilled water instead of the sample was used as blank (A_0). The chelating ability Cu^{2+} and Fe^{2+} are expressed as $[(A_0 - A_s)/A_0] \times 100\%$.

Protective Effects against Hydroxyl Radical Induced DNA Damage. The protective effects of the Gg-AOP fractions (I and II) against DNA damage caused by hydroxyl radicals were evaluated according to the method of Arnao.[35] A reaction was induced by placing the following reagents in an Eppendorf tube: 0.5 μg of pBR322 DNA was added to the mixing solution (30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$) that contained 25 μg of Gg-AOPI and Gg-AOPII. The final volume of the mixture was brought up to 20 μL with deionized distilled water. The mixture was then incubated for 30 min at 37 °C. Finally, the mixture was subjected to 1% agarose gel electrophoresis, after which the DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide.

Statistics. Each data point represents the mean of three samples ± SD. Data were subjected to analysis of variance (ANOVA) followed by Duncan's test, and the significance level of $p < 0.05$ was employed.

RESULTS AND DISCUSSION

Isolation and Purification of Antioxidant Protein Fraction (Gg-AOP) from *G. gnemon* Seed. Isolation of antioxidant Gg-AOP fractions was tested at each purification step. The crude protein was fractionated with ammonium sulfate. The 0−30% fraction exhibited highest antioxidative activity against DPPH radicals as compared with 30−60 and 60−80% fractions, where the antioxidative activity was about 2.1-fold stronger than that of the 30−70% fraction and 1.8-fold stronger than that of the 60−80% fraction. About 30.87% of the protein material applied was recovered as shown in Table 1. The pellet was dissolved in buffer and dialyzed in nitrocellulose membrane to remove ammonium salts and other low molecular weight components such as free sugars or polyphenols, which could contribute to the antioxidant activity. The ammonium sulfate fraction was then applied onto a CM-cellulose column and diluted with buffer. Bound proteins were eluted into some protein with a linear gradient of 0.4−1.2 M ammonium acetate. The major peak protein number fraction between the 130th and 145th tubes was potent to antioxidant activity (AOP). The antioxidant activity of each fraction was evaluated using the DPPH scavenging method. The protein content of fractions was also simultaneously estimated according to the Bradford method, which corresponded with the peaks at 280 nm curves. The active fraction was then applied onto a DEAE-cellulose column by eluting with 10 mM Tris-HCl buffer (pH 6.8) with a linear gradient of 0−1 M NaCl. Fractionation of the protein resulted in two peaks (Gg-AOPI and Gg-AOPII) as monitored at 280 nm (Figure 1). The fractions falling under peak I (from 29 to 33) and peak II (from 105 to 135) displayed the maximal antioxidant activity, and they were designated active peaks. The fractions of each peak were pooled separately and concentrated.

The yields of their fractions and antioxidant activities are shown in Table 1. The scavenging effects of each fraction on the DPPH free radicals were evaluated. DPPH was the most effective and extensive method for evaluating the activity of antioxidants to scavenge the free radicals.[19,36,37] The results showed that the antioxidant activities with specific activity of the Gg-AOP on CM-cellulose step eluted by ammonium acetate were higher than the ammonium sulfate step, which indicated that the nonantioxidant material in Gg-AOP was removed in this step. Two protein fractions (Gg-AOPI and Gg-AOPII) were successfully removed from Gg-AOP via DEAE-cellulose. The specific activity values of Gg-AOPI and Gg-AOPII were 12.104 ± 0.080 and 18.70 ± 0.150 mg vit-C/mg protein, respectively. Gg-AOPII showed higher radical scavenging activities than Gg-AOPI. However, Gg-AOPI and Gg-AOPII exhibited significantly higher than Gg-AOP activities on CM-cellulose step by comparison of the specific activity values, which indicated that the purification method was affected. This is similar to results that have been reported by other researchers.[36,38] The final purification step showed that AOP activity produced overall final purification yields of 0.39% (Gg-AOPI) and 0.825% (Gg-AOPII). The purities of Gg-AOPI and Gg-AOPII were determined by 15% SDS-PAGE gel. The two resutling fractions had the high purity of AOP. Two protein bands with molecular weights of approximately 30 and 12 kDa were found (Figure 2).

Determination of Peptide Mass Fingerprinting (PMF) and Amino Acid Sequence Analysis. The PMF of the SDS-PAGE gel bands of Gg-AOPI (30 kDa) and Gg-AOPII (12 kDa) was acquired by MALDI-TOF-MS using α-cyano-4-hydroxycinnamic

Table 1. Summary of Purification Steps of Gg-AOP Fractions[a]

step	total volume (mL)	total protein (mg)	total activity[b] (VCEAC)	specific activity (mg vitamin C/mg protein)	yield[c] (%)
crude protein	154 ± 2.0	400.10 ± 5.2	294.47 ± 4.1	0.736 ± 0.008	100.00
amonium precipitation (0−30%)	48 ± 0.5	123.50 ± 1.3	118.07 ± 1.1	0.956 ± 0.007	30.87
CM-cellulose	54 ± 1.0	21.80 ± 0.5	92.87 ± 1.8	4.264 ± 0.036	5.45
DEAE-cellulose					
fraction I (Gg-AOPI)	45 ± 2.0	1.54 ± 0.0	18.46 ± 0.8	12.104 ± 0.080	0.39
fraction II (Gg-AOPII)	95 ± 3.0	3.30 ± 0.1	61.71 ± 1.5	18.700 ± 0.150	0.83

[a] Values are the mean ± SD ($n = 3$). [b] The antioxidant activity was expressed as vitamin C equivalent antioxidant capacity (VCEAC). [c] Calculated on the basis of the total protein.

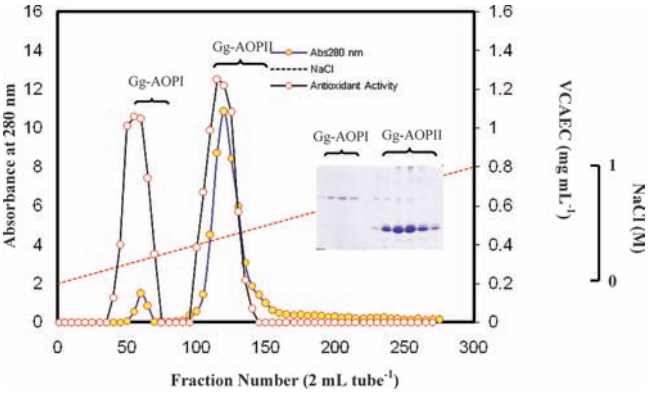

Figure 1. Elution profile of *Gnetum gnemon* antioxidant protein (Gg-AOP) onto DEAE-cellulose column, 2.5 × 75 cm; gradient elution 0−1 M NaCl. (Inset) Protein fraction profile in 15% SDS-PAGE.

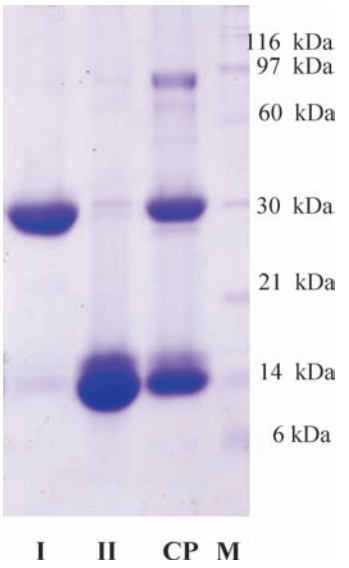

Figure 2. SDS-PAGE photograph of protein fraction Gg-AOP. Lanes: M, protein marker; CP, crude protein; I, Gg-AOPI; II, Gg-AOPII.

acid as the matrix, respectively. The MALDI-TOF spectrum of the Gg-AOPI generated from in-gel trypsin digestion is shown in Figure 3. In parallel, the MALDI-TOF spectrum of a control background-only band was also obtained, following the same in-gel trypsin digestion procedure. The PMF of Gg-AOPI is summarized in Table 2, after excluding the trypsin autolysis products from the control spectrum. It had three abundant peptide ions of m/z 2056.80, 2438.00, and 963.52. Figure 3A also shows several relatively weak peaks, including m/z 2510.00, 1219.70, 1696.70, and 2070.70. Likewise, the MALDI-TOF spectrum of the tryptic peptides of Gg-AOPII was obtained as shown in Figure 3B. The size of the peptide ions varies from m/z 904.40 to 3070.00. The most abundant peptide ions are m/z 1593.70 and 1516.70. Other relatively weak peaks contain m/z 2404.10, 1137.60, 1.898.00, and so on. On the basis of the above information, it is clear that the PMF of Gg-AOPI is distinct from that of Gg-AOPII, indicating that they are different from each other and are most likely encoded by different genes. Furthermore, analyses by the MASCOT search program suggested that there is no protein in database matching to either Gg-AOPI or Gg-AOPII.

The protein fractions of Gg-AOP were subjected to N-terminal amino acid sequencing. The N-terminal of the Gg-AOPII is determined as GNGKATVAILVKQKVQYGQQ, which is rich in glutamine (Q), glycine (G), and valine (V). We failed to identify any protein in the database by searching http://www.ncbi.nlm.nih.gov/BLAST/ with the peptide sequences described above, suggesting that Gg-AOPII is a newly discovered protein that has not been extensively characterized previously. In contrast, the N-terminal sequence of Gg-AOPI failed to be determined probably because its N-terminus was blocked. This is not surprising because the N-termini of some storage proteins from mungbean, oat, cotton, rice, and rape are reported to be blocked.[39−44]

DPPH and ABTS Radical Scavenging Activity. Antioxidant capacities of Gg-AOPI and Gg-AOPII were evaluated with the DPPH and ABTS tests. DPPH is a stable free radical that shows a maximum at 517 nm in ethanol and is widely used to evaluate the antioxidant activity of natural compounds.[14,19,45,46] The DPPH radical scavenging activity indicates the ability of the antioxidant compound to donate electrons or hydrogens, thereby converting to a more stable molecule with reduced absorbance.[26] On the basis of this principle, the antioxidant activity of a substance can be expressed as its ability to scavenge the DPPH radical. The effects of different concentrations of Gg-AOP fractions on the scavenging activities of DPPH radicals with spectrophotometry are shown in Figure 4A. Reduced glutathione (G-SH) was used as positive control. The scavenging ability of the Gg-AOP on DPPH radical increased with increasing concentration. The IC_{50} values for DPPH radical scavenging activity were 0.021 mg mL^{-1} (Gg-AOPI) and 0.027 mg mL^{-1} (Gg-AOPII), although their scavenging abilities were lower than that of G-SH

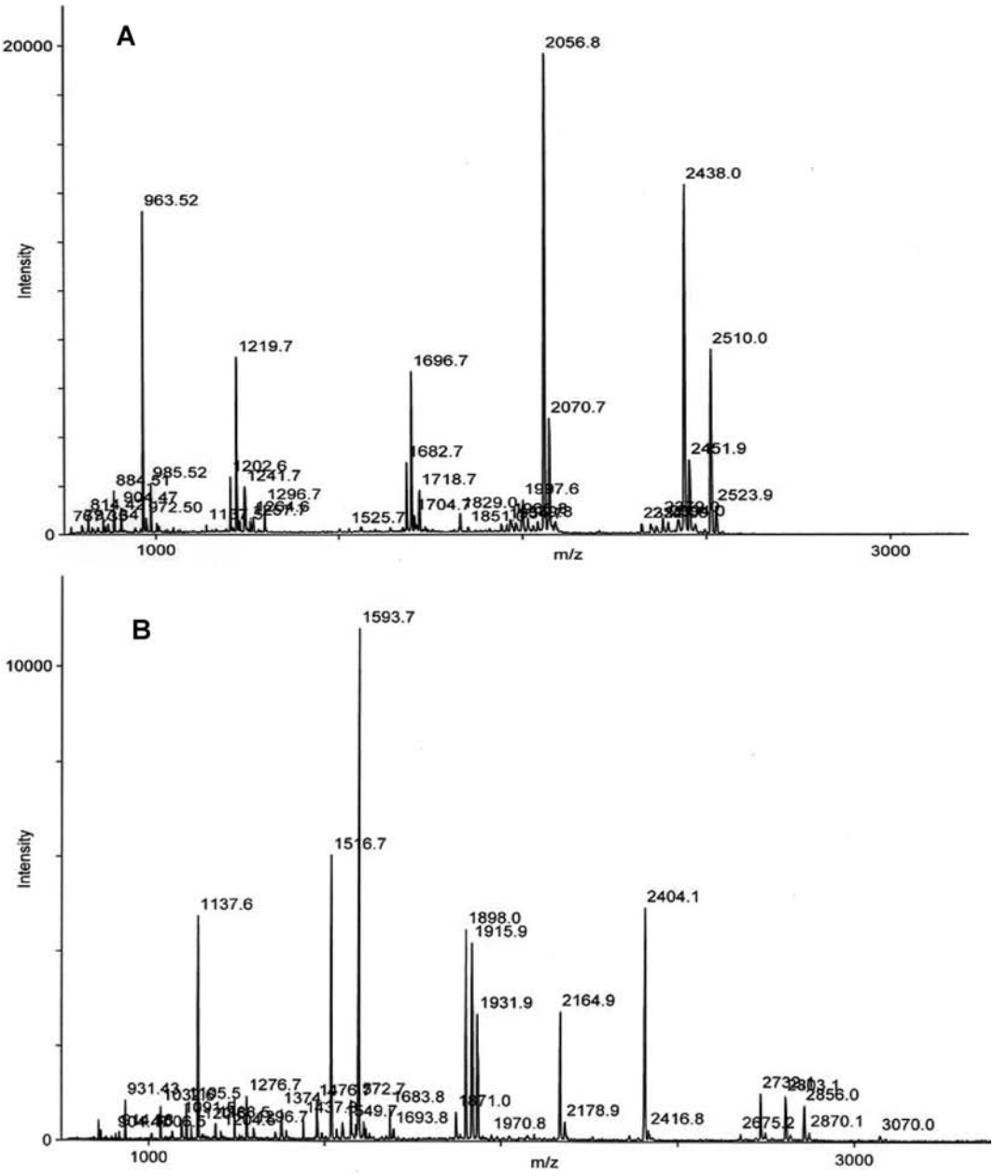

Figure 3. Comparison of the MALDI mass spectra of tryptic in-gel digests of Gg-AOPI (A) and Gg-AOPII (B). All labeled peaks denote monoisotopic peptide masses $[M + H]^+$.

(0.01 mg mL^{-1}). The results of the antioxidant capacity assay using ABTS are shown in Figure 4B. Gg-AOPI and Gg-AOPII displayed antioxidant activities as they were able to scavenge ABTS. All of the fractions displayed a positive correlation between the concentration of protein fraction added and the level of inhibition. These results show that Gg-AOPI and Gg-AOPII had almost identical effects, whereas G-SH showed higher activity at all concentrations.

Glutathione is recognized to be a potent antioxidant, and the activity is attributed to the sulfhydryl group of cysteine; therefore, cysteine-containing peptides could be considered as effective scavengers of DPPH and ABTS radicals. Furthermore, Kim et al.[47] reported that several amino acids such as His, Pro, Ala, and Leu contribute to the scavenging of free radicals. The amino acid composition of the Gg-AOP fractions in the present study is shown in Table 3. Gg-AOPI was rich in Phe, His, Pro, Met, Ile, and Cys compared with the other fractions. It was demonstrated that the antioxidant activity of the Gg-AOP fractions was related to the composition of amino acids and that the abundance of the amino acids Phe, His, Pro, Met, Ile, and Cys in the Gg-AOPI fraction may correlate with its strong antioxidant activity. The results mentioned earlier revealed that Gg-AOPI and Gg-AOPII possessed the same potent activity of quenching radicals. This suggested that both Gg-AOPI and Gf-AOPII possibly contained some proton-donating peptide components and could react with free radicals to covert them to more stable products and terminate radical chain reaction.

In addition, the difference between the radical scavenging capacities for DPPH and ABTS could be, in part, due to the difference of the radicals' solubility and diffusivity in the reaction

Table 2. Mass Peaks of Gg-AOPI and Gg-AOPII Fractions for MASCOT Database Search

gel band Gg-AOPI, PMF (m/z)		gel band Gg-AOPII, PMF (m/z)	
768.35	1696.70	904.40	1683.80
778.34	1704.70	914.10	1693.80
814.42	1718.70	931.43	1871.00
884.51	1829.00	1006.50	1898.00
904.47	1851.10	1031.10	1915.90
963.52	1934.60	1091.50	1931.90
972.50	1966.00	1137.60	1970.80
985.52	1997.60	1201.60	2164.90
1137.52	2056.80	1204.60	2178.90
1202.60	2070.70	1276.70	2404.10
1219.70	2223.30	1296.70	2416.80
1241.70	2250.80	1374.70	2675.20
1257.70	2379.00	1437.60	2732.10
1264.60	2438.00	1476.70	2803.10
1296.70	2451.90	1516.70	2856.00
1525.70	2510.00	1572.70	2870.10
1682.70	2523.90	1593.70	3070.00

medium. Although DPPH scavenging is a widely used method for the assessment of free radical scavenging activity of natural products, it has a notable limitation when used to interpret the role of hydrophilic antioxidants because DPPH can be dissolved only in organic media (especially in alcoholic media), not in aqueous solutions.[33,35] A further disadvantage is that DPPH serves both as oxidizing substrate and as the reaction indicator molecule; therefore, the assay would easily lead to the problem of spectral interferences.[45] In contrast, ABTS can be solubilized in aqueous as well as organic media; thus, radical scavenging activities of both hydrophilic and lipophilic compounds can be measured.[35] The results suggested that the ABTS method was more sensitive than the DPPH assay for the measurement of antioxidant activity of water-soluble proteins in an aqueous solution. A similar observation was reported for fermented shrimp biowaste, which exhibited 40% scavenging activity for DPPH at a concentration of 1.0 mg/mL, compared with a 95% activity against ABTS even at a concentration of 0.5 mg/mL.[46]

Superoxide Radical Scavenging Activity and Lipid Peroxidation Inhibition in Linoleic Acid Model System. Superoxide radical is one of the strongest free radicals in cellular oxidation reactions because it further produces various kinds of cell-damaging free radicals and oxidizing agents.[48] The superoxide radical scavenging activity of Gg-AOPs was evaluated by measuring the inhibition of pyrogallol autoxidation that is catalyzed by the superoxide radical. Gg-AOPII exhibited the higher activity, about 67.34% at a concentration of 0.1 mg/mL compared with Gg-AOPI (61.95%) (Figure 5A). The antioxidant activity of Gg-AOPs was measured against linoleic acid emulsion system during incubation times of 0−8 days at 40 °C. The inhibition on autoxidation of linoleic acid by the Gg-AOP was expressed as the relative antioxidant activity based on the induction period using the ferric thiocyanate method.[30,49] A longer induction period on linoleic acid peroxidation indicates a stronger antioxidant activity. As shown in Figure 5B, the autoxidation of linoleic acid was effectively inhibited by the addition of Gg-AOPs. The induction periods increased from 2 to 8 days as the control, but Gg-AOP (I or II) was close to that of BHT. This was presumed to

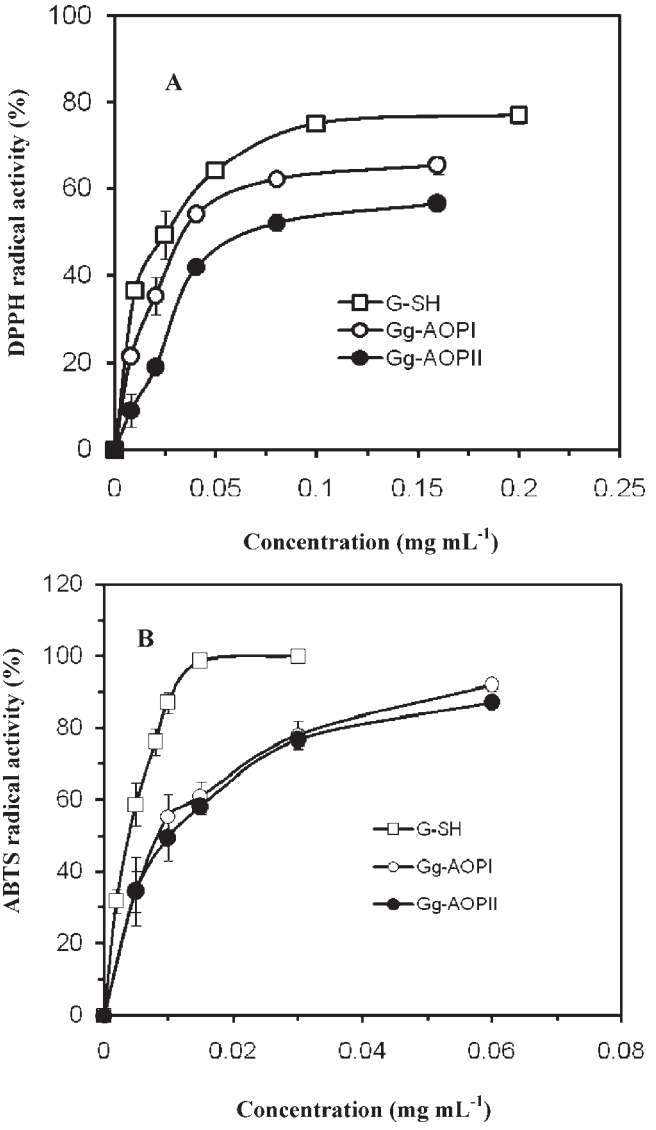

Figure 4. DPPH (A) and ABTS (B) radical scavenging effects of protein fraction Gg-AOPs at various concentrations.

be because Gg-AOPs react with radicals in the system including peroxyl radicals and thereby inhibit the propagation cycle of lipid peroxidation. The profiles of the amino acid compositions of Gg-AOPI and Gg-AOPII (Table 3) show that the Gg-AOPs are rich in hydrophobic amino acids residues. Therefore, the higher antioxidative potency of this protein can be expected because of the larger hydrophobicity properties, which may lead to a higher interaction between the protein and fatty acids. Moreover, the activity of histidine-containing protein has also been reported to act against lipid peroxidation.[49] This activity has been suggested due to the presence of an imidazole ring in histidine structure, which may involve in hydrogen donation a lipid radical trapping ability. Therefore, we can speculate that the N-terminal histide may contribute higher antioxidative activity to the Gg-AOP sequence.

Reducing Power and Metal Chelating Activity. Reducing power is a measurement that provides an estimate of a compound's ability to reduce ferric iron(III) to ferrous iron(II) and is determined using a redox-linked colorimetric reaction.[50] The reducing capacity of a compound may serve as a significant

Table 3. Amino Acid Composition of Protein Fractions (Gg-AOPI and Gg-AOPII) from *Gnetum gnemon*[a]

amino acid	Gg-AOPI	Gg-AOPII
aspartic acid (Asp)	11.39	13.65
threonine (Thr)	7.84	6.68
serine (Ser)	10.59	5.56
glutamic acid (Glu)	10.40	13.94
proline (Pro)	2.84	1.93
glycine (Gly)	7.69	9.40
alanine (Ala)	7.43	5.36
cysteine (Cys)	0.39	0.08
valine (Val)	7.46	9.02
methione (Met)	1.25	0.01
isoleucine (Ile)	5.81	4.18
leucine (Leu)	9.12	8.25
tyrosine (Tyr)	3.87	7.03
phenylalanine (Phe)	3.25	0.94
histidine (His)	0.78	0.36
lysine (Lys)	3.93	8.42
arginine (Arg)	4.61	1.53
tryptophan (Trp)	1.38	2.68

[a] %, mol base.

indicator of its potential for use as an antioxidant.[51] In addition, samples with higher reducing power have better abilities to donate electrons or hydrogens. The reducing power of the protein isolated (Gg-AOP) is shown in Table 4; increasing reducing power activity (expressed as equivalents of vitamin C) indicates greater reducing power potency, and Gg-AOPI showed better reducing power than Gg-AOPII. This suggests that Gg-AOPI was a more potent antioxidant than Gg-AOPII, which agrees with the radical scavenging activity. Also, the result indicated that reduction-related products appeared to be present in Gg-AOP. Transition metals, such as Fe^{2+} and Cu^{2+}, can catalyze the generation of ROS such as the hydroxyl radical and superoxide anion.[52] This metal generates hydroxyl radical according to Fenton's reaction, by which the lipid peroxidation chain reaction is accelerated. As a result, the chelation of metal ions contributes to antioxidant activities.

The chelating activities of Gg-AOPI and Gg-AOPII were measured using Cu^{2+} and Fe^{2+}. Gg-AOPI showed higher chelating activities on Cu^{2+} and Fe^{2+} than Gg-AOP II at the same concentration. The results obtained from this study showed that Gg-AOPs demonstrated binding capacities on Cu^{2+} and Fe^{2+}. As shown in Table 4, the chelating activities of both Gg-AOP fractions on Cu^{2+} and Fe^{2+} were significantly different. This difference may be caused by differences in the structure and length of the peptides and amino acid compositions.

Inhibition of DNA Strand Breaks by Gg-AOPI and Gg-AOPII. In this work, we also evaluated the oxidative DNA damage protective activity of the isolated protein against hydroxyl radical induced DNA damage on pBR322 plasmid DNA, by an in vitro method. In the case of plasmids, a single-strand break in supercoiled (SC) plasmid DNA, exposed to hydroxyl radical derived from the Fenton reaction, leads to the formation of open circular (OC) DNA. As shown in Figure 6, the incubation of pBR322 plasmid DNA with Fenton's reagent for 20 min gave rise to cleavage of SC to make the OC form in line 3, indicating that the hydroxyl radical generated by the Fenton reaction produced

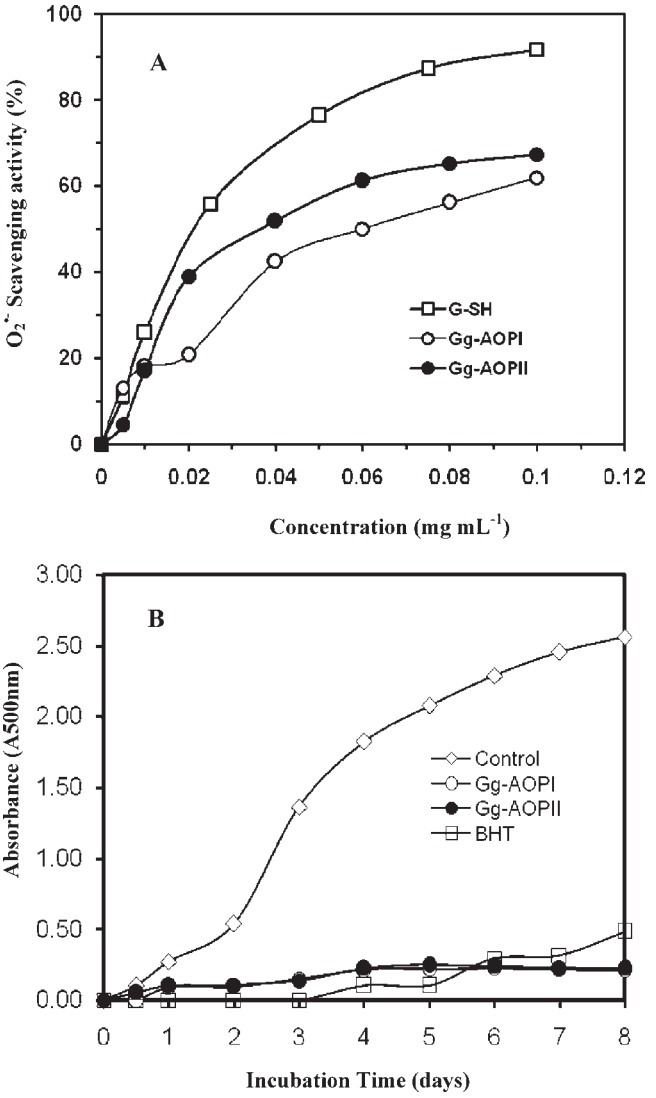

Figure 5. Superoxide (A) and lipid peroxidation inhibitory (B) activity of protein fraction Gg-AOPs. The activity was measured in a linoleic acid oxidation system for 8 days. Butylated hydroxytoluene (BHT) and glutathione (G-SH) were used as positive controls to compare the activity protein fraction.

Table 4. Reducing Power and Metal Ion Chelating Activity of Gg-AOP Fractions*

	chelating (%)		
	Cu^{2+}	Fe^{2+}	reducing power (mg vitamin C)
Gg-AOPI	65.57 ± 0.98 a	98.42 ± 0.53 a	0.213 ± 0.021 a
Gg-AOPII	27.42 ± 0.84 b	20.93 ± 2.37 b	0.150 ± 0.039 a

*Values are the mean ± SD (n = 3). Values in the same column with different letters were significantly different ($p < 0.05$). The concentrations of samples were 0.5 mg mL^{-1}.

single-strand DNA breaks. However, the addition of the isolated protein (Gg-AOPI and Gg-AOPII) at 25 μg to the DNA and Fenton's reagent mixture significantly decreased the conversion of SC DNA to OC DNA. DNA is another sensitive biotarget for

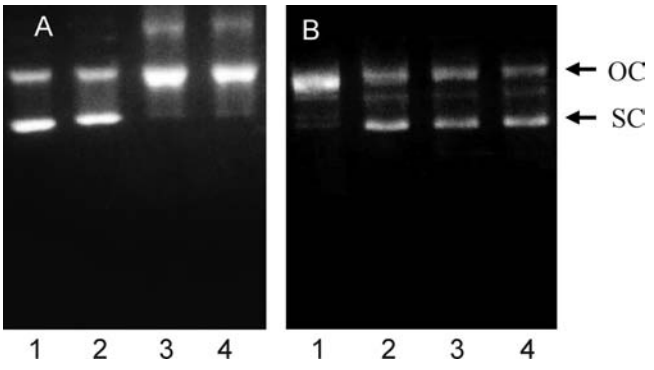

Figure 6. Inhibitory effect of protein fraction Gg-AOPs on DNA nicking induced by hydroxyl radicals. Each reaction solution (20 μL) contained 30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$. The DNA nicking was initiated by mixing 0.5 μg of pBR322 plasmid DNA with the reaction solution for 5, 10, 20, and 30 min (lanes 1, 2, 3, and 4, respectively) (A) and control (30 min), Gg-AOPI, Gg-AOPII, and G-SH (lanes 1, 2, 3, and 4, respectively) (B) at 37 °C. The reaction was stopped by adding 4 μL of the loading buffer. SC, supercoiled; OC, open circular.

ROS-mediated oxidative damage.[53,54] DNA damage by ROS can initiate carcinogenesis or contribute to the pathogenesis of neurodegenerative conditions such as Parkinson's and Alzheimer's diseases. The hydroxyl radical is one kind of ROS that is recognized as a DNA-damaging agent of physiological significance.[55] DNA single-strand cleavage has also been reported in other free radical involved circumstances, such as γ-radiation and peroxyl radical.[56,57] The protection ability of the isolated proteins was related to their scavenging of H_2O_2 and hydroxyl radical and to their chelating activities toward Fe^{2+} as shown in Table 4. These activities lead to the inhibition of the Fenton reaction and, therefore, protect the SC DNA from hydroxyl radical induced strand breaks.

In conclusion, two antioxidant protein fractions were isolated from *G. gnemon* seed with molecular weights of approximately 30 kDa (Gg-AOPI) and 12 kDa (Gg-AOPII). The result showed that two protein fractions exhibited significant ($p < 0.05$) antioxidant activities against free radicals such as DPPH, ABTS, and superoxide anion and showed activities similar to those of glutathione and BHT in a linoleic acid emulsion assay system. Moreover, Gg-AOPI and Gg-AOPII also exhibited notable reducing power and strong chelating effect on Fe^{2+} and protected hydroxyl radical induced oxidative DNA damage. The data obtained by the in vitro systems obviously established the antioxidant potency of Gg-AOP from melinjo (*G. gnemon*) seed.

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