JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Rice Albumin N-Terminal (Asp-His-His-Gln) Prevents against Copper Ion-Catalyzed Oxidations

CHUNHUA WEI,† SU DUY NGUYEN,† MEE REE KIM,‡ AND DAI-EUN SOK*,†

College of Pharmacy and Department of Food and Nutrition, Chungnam National University, Gung-Dong 220, Yuseong-Gu, Taejon 305-764, Korea

Chromatographic separation of soluble proteins from rice (*Oryza sativa* L.) yielded a major albumin protein (16 kDa), with the DHHQVYSPGEQ sequence in the N terminus, showing antioxidant action. The rice albumin was more potent than other rice proteins in preventing Cu^{2+}-induced low-density lipoprotein (LDL) oxidation. Additionally, it also exhibited a remarkable suppression of HOCl oxidation. In a further study, albumin inhibited Cu^{2+}-induced oxidation of LDL in a stoichiometric manner with an EC_{50} value of 4.3 μM, close to that of serum albumins. Moreover, after digestion with trypsin or chymotrypsin, it maintained its antioxidant action. In an experiment to see the involvement of the N terminus in antioxidant action, a synthetic tetrapeptide, equivalent to the N terminus DHHQ, was found to inhibit Cu^{2+}-induced LDL oxidation or degradation of apolipoprotein B, similar to that of rice albumin. In mechanistic analyses, the action of rice albumin or tetrapeptide is primarily due to the removal of Cu^{2+}, as suggested from its inhibitory effect on Cu^{2+}/diphenylcarbohydrazide (DPCH) complex formation. However, despite its similar inhibitory effect on Cu^{2+}-induced oxidation of LDL, rice albumin was less effective than serum albumin in inhibiting Cu^{2+}/DPCH complex formation, suggesting that the number of Cu^{2+}-binding sites in rice albumin may be less than that in serum albumins. Taken together, rice albumin exerts a potent preventive action against Cu^{2+}-induced oxidations, which is due to the Cu^{2+} binding by DHHQ in the N-terminal sequence. Such a role as a Cu^{2+} chelator would add up to the application of rice albumin protein.

KEYWORDS: Rice albumin; copper oxidation; Cu^{2+} chelator; HOCl; DHHQ

INTRODUCTION

Reactive oxygen or nitrogen species are known to be triggers of various degenerative or pathological processes such as aging, inflammation, carcinogenesis, and degenerative heart or brain diseases (*1*−*3*). There have been many reports concerning plant polyphenols with regard to their antioxidant actions against such reactive oxygen/nitrogen species in vitro (*1*, *3*−*5*). Especially some edible plants, vegetables, or fruits contain polyphenol components possessing remarkable antioxidant actions (*6*−*8*). In addition, antioxidant actions are also expressed by storage proteins from sweet potato root (*9*), potato tuber (*10*), or yam tuber (*11*).

Rice is known to contain major proteins such as glutelin, prolamin, globulin, or albumin, among which albumin (14−16 kDa) and α-globulin are known to be major rice allergens (*12*, *13*). Rice albumin, a water-soluble protein, constitutes about 2−5% (dry weight) of total rice protein (*14*). Interestingly, the N-terminal amino acid sequence of rice albumin is homologous to that of human serum albumin (HSA) (*12*): 14 kDa rice albumin (DHHK) or 16 kDa rice albumin (DHHQ) vs HSA (DAHK). HSA is a unique protein; it maintains colloidal osmotic pressure in the vasculature and has a number of important functional properties (*15*). It strongly binds fatty acids, some drugs, and drug metabolites and has a number of cation and anion binding sites (*15*−*17*). Previous studies have demonstrated that the four amino acids occupying the N terminus of HSA—aspartate, alanine, histidine, and lysine (DAHK)—constitute a relatively high-affinity binding site for a number of cations, specifically nickel, cobalt, and copper (*17*, *18*). Furthermore, in addition to DAHK, the peptides containing His residue at the second or third position from the N-terminal were observed to exhibit a strong affinity for copper ions and prevent copper-induced oxidation of low-density lipoproteins (LDLs) (*19*, *20*). It is then conceivable that the rice albumin bearing a His residue at the second or third position from the N-terminal could also express an antioxidant action against copper ion-catalyzed oxidation or other oxidant radicals.

We report here that the purified rice albumin (16 kDa) and the tetrapeptide (DHHQ), corresponding to the amino acids present in the N terminus of 16 kDa albumin, have a potent antioxidant action, similar to that of DAHK in preventing against copper-induced oxidation.

* To whom correspondence should be addressed. Tel: +82-42-821-5940. Fax: +82-42-823-6566. E-mail: daesok@cnu.ac.kr.
† College of Pharmacy.
‡ Department of Food and Nutrition.

10.1021/jf062387g CCC: $37.00
Published on Web 02/17/2007

MATERIALS AND METHODS

Chemicals. Reagents including diphenylcarbohydrazide (DPCH) and HOCl (Na-hypochlorite) were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Thiobarbituric acid (TBA) and DEAE cellulose (DE52) were obtained from Lancaster Chemical Co. (Eastgato, White Land, Morecamble, England) and Whatman Co. (Brentford Middlesex, United Kingdom), respectively. Hydrogen peroxide (33%) was from Wako Pure Chemicals Industry (Osaka, Japan). Asp-Ala-His-Lys (DAHK) was obtained from Bachem Co. (Bubendorf, Switzerland), and Asp-His-His-Gln (DHHQ) and Asp-His-His-Lys (DHHK) were from AnyGen Co., Ltd. (Gwangju, Korea). Protein markers were procured from Technical Frontier Co. (Tokyo, Japan). Metal ions and other reagents were of analytical grade.

Albumin Extraction and Purification. Rice (*Oryza sativa* L.) grains, produced in Cheolwon province, Korea, were ground and defatted with five-fold cold acetone. Then, the power of defatted rice was incubated with 4 volumes of distilled water under stirring (1 h), and then, the supernatant, obtained from centrifugation (10000*g* × 15 min) of the above mixture, was subjected to 70% ammonium sulfate precipitation as described before (*12*). The precipitate, after extensive dialysis against 20 mM Tris-HCl buffer (pH 8.6), was loaded onto a DEAE cellulose column (3 cm × 40 cm), which was eluted with a linear gradient of NaCl (0−0.1 M in 20 mM Tris buffer) as described previously (*12*, *13*). The fractions (5 mL), containing 16 kDa protein, were pooled, dialyzed (H_2O), concentrated, and loaded onto a Sephacryl S-100 HR (1.4 cm × 100 cm) column, which was eluted with 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. The fractions, containing 16 kDa albumin, were collected, dialyzed (H_2O), and lyophilized for further study. The amount of protein was determined by Lowry method (*21*) and bicinchoninic acid method (*22*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of Rice Albumin. The albumin sample was dissolved in 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with 5% 2-mercaptoethanol, heated in boiling water for 5 min, and then subjected to 15% acrlyamide SDS-PAGE analysis according to the method of Laemmli (*23*). Coomassie brilliant blue R-250 was used for protein staining in PAGE analysis.

Determination of Amino Acid Sequence in N Terminus. The N-terminal amino acid sequence of pure protein was determined by standard Edmann degradation on a model 476A Automatic Protein Sequencer (Applied Biosystems Inc., Foster City, CA) at the Korea Basic Science Institute (Daejon, Korea).

Preparation of Trypsin- or Chymotrypsin-Treated Albumin. Trypsin- or chymotrypsin-treated albumin was prepared by exposing rice albumin (0.16 mg/mL) to trypsin (16 units/mL) or chymotrypsin (10 units/mL) in 10 mM phosphate-buffered saline (PBS) (pH 7.4) for 1 h and inactivating the enzyme by heating the mixture in boiling water for 30 min.

Prevention by Protein or Peptide against Cu^{2+}-Induced LDL Oxidation. LDL (0.1 mg of protein/mL), prepared by sequential ultracentrifugation of human plasma (*24*), was incubated with 5 μM Cu^{2+} in the presence of rice albumin (5 or 10 μM) in 10 mM PBS buffer (pH 7.4) at 37 °C for 3 h, and the formation of thiobarbituric acid-reactive substances (TBARS) was measured as previously described (*25*−*27*). LDL was incubated with 5 μM Cu^{2+} in the presence of albumin or peptide at various concentrations (0.125−20 μM). The protective activity (%) of protein or peptides against Cu^{2+}-induced LDL oxidation was calculated according to the following equation: protection (%) = $[1 - (A_p - A_b)/(A_c - A_b)] \times 100$, where A_p is the absorbance at 532 nm in the presence of Cu^{2+} and protein or peptide, A_c is the absorbance in the presence of Cu^{2+} only, and A_b is the absorbance in the absence of Cu^{2+}. EC_{50} was expressed as the concentration of protein or peptide to achieve 50% protection.

Protection by Albumin against AAPH-Induced LDL Oxidation. LDL (0.1 mg of protein/mL) was incubated with 5 mM AAPH in the presence of albumin (5 or 10 μM) in 10 mM PBS buffer (pH 7.4) at 37 °C for 3 h, and the formation of TBARS was measured as described above (*28*).

Prevention by Albumin against Fe^{3+}/Ascorbate-Induced Formation of Hydroxyl Radicals. The protective action of albumin against Fe^{3+}-induced formation of hydroxyl radicals was assessed using 2-deoxyribose decomposition method (*29*). Purified rice albumin (5 or 10 μM) was included in the final volume of 1.0 mL of 10 mM phosphate buffer (pH 7.4) containing 20 μM $FeCl_3$, 0.1 μM EDTA, 1.42 mM H_2O_2, and 2.8 μM 2-deoxyribose, and the oxidation was started by adding 100 μM ascorbic acid. After 60 min of incubation at 37 °C, the formation of TBARS was measured at 532 nm as previously described (*27*). The protective action of albumin was determined as described in LDL oxidation.

Prevention by Rice Albumin or Peptides against HOCl-Induced Inactivation of Acetylcholinesterase. Eel acetylcholinesterase (0.25 U/mL) was incubated with 10 μM HOCl in 0.1 mL of PBS buffer (pH 7.4) at 37 °C in the presence of albumin (5 or 10 μM), and 15 min later, an aliquot of 20 μL was used to determine the remaining activity of acetylcholinesterase, which was monitored at 412 nm (*30*). The protection (%) was calculated according to the equation: protection (%) = $(A_p - A_b)/(A_c - A_b) \times 100$, where A_p is activity in the presence of HOCl and protector (albumin or peptide), A_c is the activity in the presence of protector only, and A_b is the activity in the presence of HOCl only.

For the inclusion of HOCl at designated concentrations, the NaOCl solution (Sigma Chemical Co.) was first diluted with PBS buffer to produce 100 μM solution, which was finally diluted to 10 μM in the final reaction mixture.

Protective Effect of Albumin or Peptide on the Degradation of Apolipoprotein B-100. LDL (0.1 mg of protein/mL) was incubated with Cu^{2+} (5 μM) in the presence of protein or peptide (0.625−20 μM) in 10 mM PBS buffer (pH 7.4) at 37 °C for 3 h, and the aliquot (10 μL) was subjected to 7.5% acrylamide SDS-PAGE analysis (*31*).

Inhibitory Effect of Albumin or Peptide on Formation of Cu^{2+}/DPCH Complex. The interaction of Cu^{2+} with rice albumin or tetrapeptide was assessed using DPCH as a metal colorimetric detector (*32*). Rice albumin or peptide (0.5−8 μM) was incubated with 2 μM Cu^{2+} in 0.5 mL of 10 mM PBS buffer (pH 7.4) at 38 °C, and 30 min later, DPCH (0.5 mM) was included in the mixture. After another 10 min of incubation, the absorbance at 495 nm, indicating the DPCH complex formation, was determined against a test inhibitor and DPCH-containing blank. The inhibitory effect was calculated according to the equation: inhibition (%) = $[1 - (A_i - A_b)/(A_c - A_b)] \times 100$, where A_i is the absorbance at 495 nm in the presence of Cu^{2+}, DPCH, and inhibitor (protein or peptide); A_c is the absorbance in the presence of Cu^{2+} and DPCH; and A_b is the absorbance in the presence of DPCH and inhibitor. Data are expressed as a mean ± SD (bar) value of triplicate experiments.

Statistical Analyses. All data are presented as means ± standard deviations (SDs) of three independent experiments. Statistical analysis was done using Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Purification of Albumin from Rice Protein. To examine the possible antioxidant action of rice protein, each protein fraction containing prolamin, glutelin, globulin, or albumin (*33*) was examined for antioxidant action in copper-induced oxidation of LDL. Because the albumin fraction showed a remarkable protection, albumin was purified from a water-soluble fraction of rice protein by ammonium sulfate fractionation and chromatographies. As shown in **Figure 1**, several protein peaks with a range of molecular masses, corresponding to albumin proteins (*12*, *13*), appeared in the profile of DEAE chromatography. The protein from peak a, containing 16 kDa albumin, was concentrated by freeze drying and then subjected to further purification by Sephacryl S-100 HR gel chromatography, which gave rise to peak b as a major one (**Figure 1**, inset). In SDS-PAGE analysis, the protein from peak b was observed to contain a single protein molecule with a molecular mass of 16 kDa

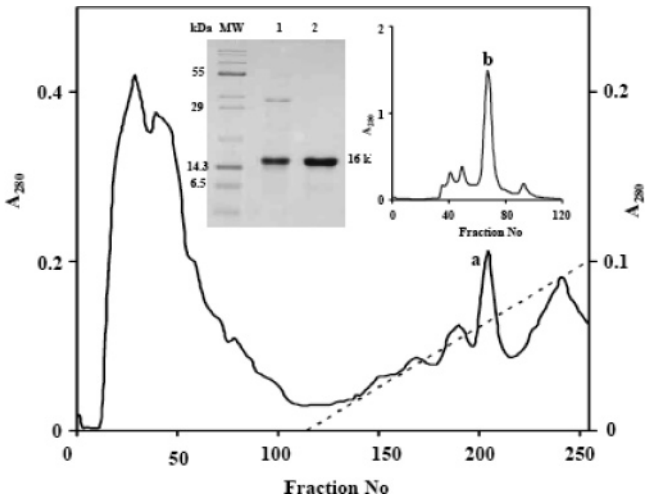

Figure 1. Purification of rice albumin by chromatography. The albumin sample, obtained from 70% ammonium sulfate precipitation, was loaded onto a DEAE cellulose column, which was eluted with a linear gradient of NaCl (0–0.1 M) in 20 mM Tris buffer. Inset: The fraction (peak a) from DEAE cellulose chromatography, after concentration, was loaded onto a Sephacryl S100-HR column, which was eluted with 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. An aliquot from peak a or b was subjected to SDS-PAGE analysis: MW, non-prestained protein markers; lane 1, protein from peak a; and lane 2, protein from peak b.

Table 1. Antioxidative Activities of Rice Albumin in Various Oxidation Systems[a]

	protection (%)			
albumin	Cu^{2+}/ LDL	AAPH/ LDL	Fe^{3+}/ ascorbate	HOCl oxidation
5 μM	82.30 ± 2.52	<5	9.68 ± 0.45	79.90 ± 5.17
10 μM	97.70 ± 2.02	<5	23.9 ± 3.76	46.21 ± 0.37

[a] Each oxidation was carried out in the presence of 16 kDa rice albumin (5 or 10 μM), and the antioxidant activities were expressed as a relative protection or inhibition value (%) as described in the Materials and Methods. Values are mean ± SD (bar) values of triplicate assays.

(**Figure 1**, inset, lane 1). In a related study, the N-terminal amino acid sequence of purified rice protein (16 kDa) was identified to be DHHQVYSPGEQ and was the same as that reported for 16 kDa albumin (*12*, *34*, *35*).

In the subsequent experiment, the protective action of 16 kDa albumin was assessed using various oxidation systems, which had been used in the screening of antioxidants. For this purpose, Cu^{2+}-induced oxidation of LDL (*26*), Fe^{3+}/ascorbic-induced generation of hydroxyl radicals (*29*), AAPH-induced oxidation of LDL (*28*), or HOCl-induced oxidative inactivation of acetylcholinesterase (*36*) was carried out in the presence of 16 kDa albumin at 5 or 10 μM. As shown in **Table 1**, Cu^{2+}-induced oxidation of LDL was reduced by 82 and 98% in the presence of 16 kDa albumin at 5 and 10 μM, respectively. Meanwhile, the protective action of albumin against Fe^{3+}/ascorbic acid-induced generation of hydroxyl radicals was not remarkable: <10% at 5 μM and <25% at 10 μM. Thus, rice albumin seemed to express a specificity toward Cu^{2+}-induced oxidation rather than Fe^{3+}-promoted oxidation. Additionally, albumin failed to show a significant protection against AAPH-induced oxidation of LDL. In a separate experiment, rice albumin at 5 μM showed a remarkable protection (79%) against prevented HOCl-induced inactivation of acetylcholinesterase, which is known to be sensitive to HOCl oxidation (*36*). However, the increase of albumin concentration to 10 μM rather decreased its protective action (46%), implying that the HOCl-modified albumin may show a negative effect on acetylcholinesterase activity at high concentrations. Taken together, rice albumin was the most effective in preventing against Cu^{2+}-induced oxidation. Therefore, the protective action of albumin against Cu^{2+}-metal-catalyzed oxidation was further studied.

Protective Activity of Albumin or Peptide against Cu^{2+}-Induced LDL Oxidation. Because LDL has been routinely employed as the target for the oxidation by copper ions (*26*, *28*), which may be implicated in atherosclerosis progress (*37*), the protective action of 16 kDa albumin in Cu^{2+}-induced oxidation of LDL was assessed extensively. When LDL was incubated with 5 μM Cu^{2+} in the presence of 16 kDa albumin at various concentrations, it was found that rice albumin inhibited LDL oxidation in a concentration-dependent manner (**Figure 2A**); rice albumin at 2.5, 5, 10, and 20 μM expressed 14.2, 82.3, 97.7, and 97.9% protection, respectively. The EC_{50} value of 16 kDa albumin in preventing against Cu^{2+}-induced LDL oxidation was estimated to be 4.3 μM, close to that of HSA (EC_{50}, 3.5 μM) or bovine serum albumin (BSA) (EC_{50}, 3.8 μM). Thus, it is interesting to observe that the protective action of rice albumin in inhibiting Cu^{2+}-catalyzed oxidation is similar to that of HSA or BSA. Because a previous study (*19*) showed that four amino acids occupying the N terminus of HSA—aspartate, alanine, histidine, and lysine (DAHK)—were responsible for the prevention against Cu^{2+}-induced oxidation of LDL, it was supposed that the N-terminal amino acid sequence (DHHQ) of rice albumin might be implicated in the inhibition of Cu^{2+}-induced oxidation of LDL. In this relation, the tetrapeptides, DHHQ, corresponding to the four amino acid residues present in N-terminal of rice albumin, and its analogue (DHHK) were synthesized and tested for the protective action against LDL oxidation. As shown in **Figure 2B**, it was found that DHHQ at 5 μM exhibited an almost complete protection against Cu^{2+}-induced LDL oxidation, analogous to the finding with DAHK derived from HSA (*19*). The EC_{50} of DHHQ, DHHK, and DAHK was estimated to be 4.46, 4.29, and 4.41 μM, respectively. Thus, the protective potency of the tetrapeptides in preventing against Cu^{2+}-induced LDL oxidation was quite similar among them. These are in a good agreement with the idea that His residue at the second or third position shows a high affinity for Cu^{2+} (*19*, *20*). Noteworthy, the potency of tetrapeptides as antioxidants was close to that of rice or serum albumins. To see the possible protective role of rice albumin in Cu^{2+}-induced oxidation in a gastrointestinal model system, the rice albumin, exposed to intestinal digesting enzymes such as trypsin or chymotrypsin, was tested for the protective action against Cu^{2+}-induced oxidation of LDL. As shown in **Figure 2C**, albumin digested with trypsin or chmotrypsin also inhibited Cu^{2+}-induced oxidation of LDL in a dose-dependent manner with a remarkable protection (>80%) at 5 μM, quite similar to the finding with intact rice albumin or DHHQ. Moreover, the trypsin-treated albumin showed a remarkable protection (>65%) at a 1:2 molar ratio of protein and Cu^{2+}. These results indicate that 16 kDa rice albumin treated with trypsin or chmotrypsin can exert a remarkable protective action against Cu^{2+}-induced oxidation under the intestinal environment.

Protective Action of 16 kDa Albumin or Tetrapeptide against Cu^{2+}-Induced Degradation of Apolipoprotein B in LDL. In an additional experiment to further elucidate the protective action of rice albumin against Cu^{2+}-induced oxidation of LDL, we examined the effect of albumin on Cu^{2+}-induced degradation of apolipoprotein B with a Cu^{2+}-binding site.

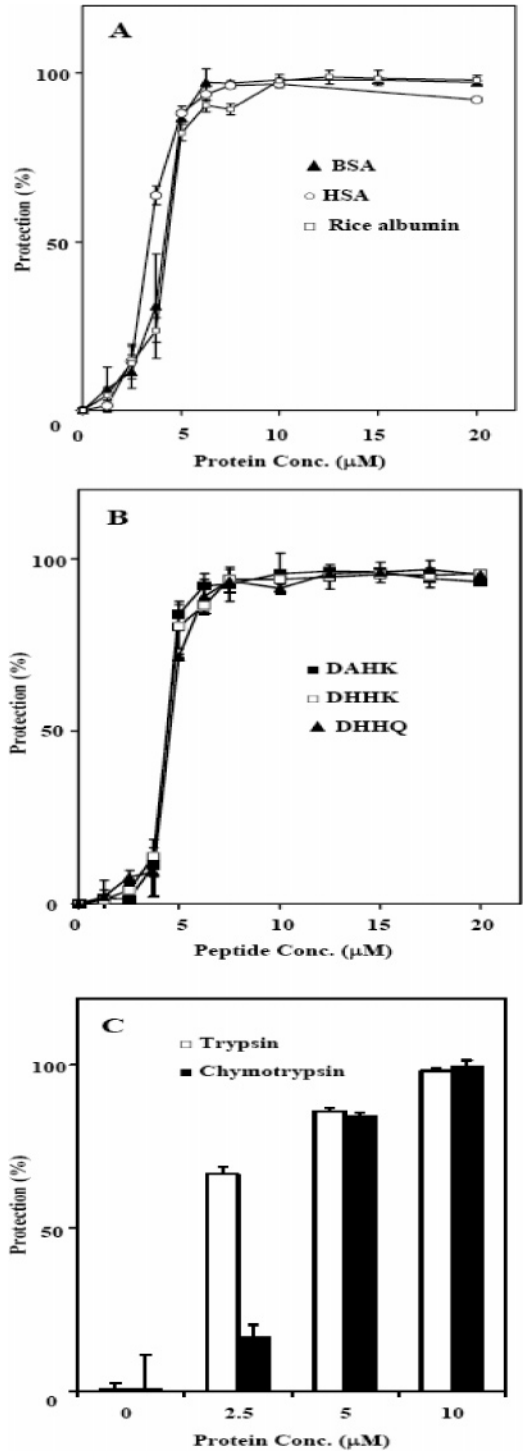

Figure 2. Prevention by protein or peptide against Cu^{2+}-induced oxidation of LDL. LDL (0.1 mg protein/mL) was incubated with 5 μM Cu^{2+} in the presence of albumin (**A**), peptide (**B**), or enzyme-hydrolyzed rice albumin (**C**) in 200 μL of 10 mM PBS buffer (pH 7.4) at 37 °C for 3 h, and then, a 0.2 mL aliquot was taken for determination of the TBARS value as described in the Materials and Methods. Data are expressed as a mean ± SD (bar) values of triplicate experiments.

Previous studies (*38*, *39*) indicated that the degradation of apolipoprotein B would be an earlier sign of Cu^{2+}-induced oxidation of LDL. Therefore, it was supposed that the inhibition of apolipoprotein B degradation might lead to the prevention against Cu^{2+}-induced oxidation of LDL. In this respect, Cu^{2+}-induced oxidation of LDL was examined in the presence of 16 kDa albumin, and the protective effect of albumin on the degradation of apolipoprotein B was analyzed by SDS-PAGE. **Figure 3A** indicated that 16 kDa albumin successfully prevented the degradation of apolipoprotein B during Cu^{2+}-induced oxidation of LDL. An almost full protection was observed with 16 kDa albumin at 5 μM, reaffirming the equivalent interaction between albumin and Cu^{2+}. In further studies (**Figure 3B**−**D**), the synthetic peptides (DAHK, DHHK, and DHHQ) at 5 μM fully prevented oxidative degradation of apolipoprotein B in LDL. From this, it is suggested that a stoichiometric interaction between copper ion and rice albumin seems to be commonly responsible for the prevention against degradation of apolipoprotein B as well as oxidation of LDL lipid.

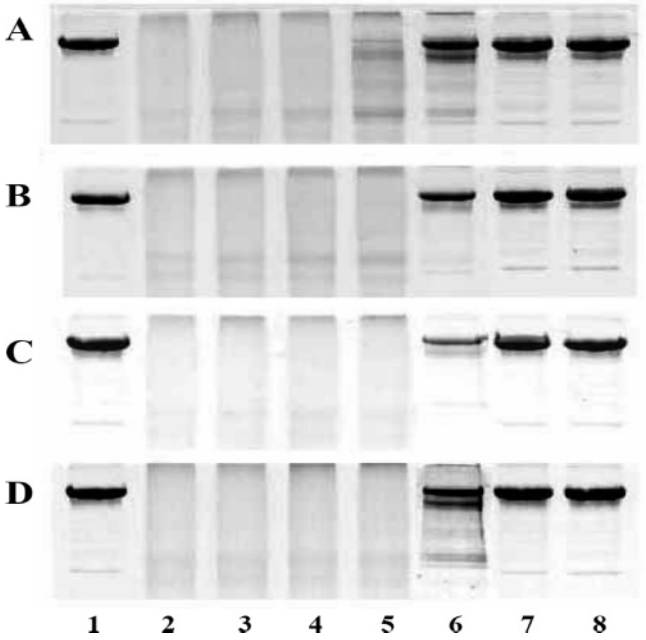

Figure 3. Protective effect of albumin or peptide on the degradation of apolipoprotein B-100. LDL (0.1 mg of protein/mL) was incubated with Cu^{2+} (5 μM) in the presence of rice albumin (**A**), DAHK (**B**), DHHK (**C**), or DHHQ (**D**) in 10 mM PBS buffer (pH 7.4) at 37 °C for 3 h. Then, the aliquot (10 μL) was taken and subjected to SDS-PAGE analysis (7.5% acrylamide): lane 1, control without Cu^{2+}; lane 2, with Cu^{2+} only; lanes 3, 4, 5, 6, 7 or 8, 0.625, 1.25, 2.5, 5, 10, or 20 μM albumin or peptide in combination with Cu^{2+}.

Cu^{2+}-Binding Capability of Rice Albumin and Synthetic Tetrapeptides. To explain the type of the interaction between Cu^{2+} and albumin protein, the ability of albumin to associate with Cu^{2+} (2 μM) was examined in the presence of DPCH (0.5 mM), which was known to form a complex with free Cu^{2+}. In this study, the formation of the Cu^{2+}:DPCH complex was examined in the presence of albumin or tetrapeptides at various concentrations. As demonstrated in **Figure 4A**, rice albumin (16 kDa) interfered with the formation of the Cu^{2+}/DPCH complex in a dose-dependent manner, showing a maximal inhibition (85.6% inhibition) at a 1:2 molar ratio of Cu^{2+}:rice albumin. In comparison, the Cu^{2+}-binding capacity of rice albumin was less than that of HSA or BSA, which exhibited somewhat greater inhibition of the Cu^{2+}/DPCH complex formation. The difference of Cu^{2+}-binding capacity between rice albumin and serum albumin may be caused by the size of protein molecule; the MW of BSA or HSA is approximately four times greater than that of rice albumin (*12*, *15*). Our recent studies (*19*, *20*) indicated that the Cu^{2+}-binding affinity of peptides was dependent on the number of Cu^{2+}-binding sites in the structure of peptides. Therefore, it is likely that greater Cu^{2+}-binding

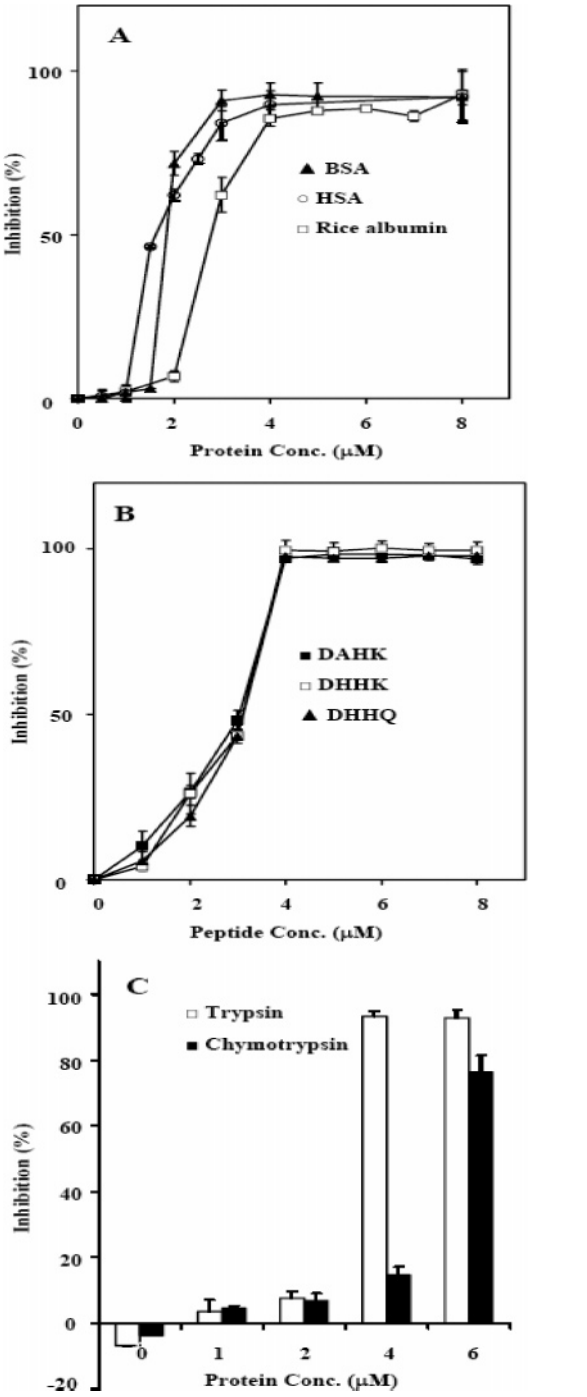

Figure 4. Inhibitory effect of albumin or peptide on formation of Cu^{2+}/DPCH complex. Cu^{2+} (2 μM) was incubated in 0.5 mL of 10 mM PBS buffer (pH 7.4) at 37 °C in the presence of rice albumin (**A**), peptide (**B**), or enzyme-hydrolyzed rice albumin (**C**) for 30 min, and then, DPCH (0.5 mM) was included in the mixture. After another 10 min of incubation, the absorbance at 495 nm was determined as described in the Materials and Methods. Data are expressed as mean ± SD (bar) values of triplicate experiments.

capacity of serum albumin may be due to the presence of another Cu^{2+}-binding site in addition to N-terminal DAHK sequence, which corresponds to a site for Cu^{2+}-binding (*20*). From these, it was assumed that two or more Cu^{2+}-binding sites might be required for the complete uptake of Cu^{2+} from the Cu^{2+}/DPCH complex. This might explain why rice protein, presumably containing one Cu^{2+}-binding site in the N terminus, inhibited the formation of the Cu^{2+}/DPCH complex effectively at a 1:2 molar ratio of Cu^{2+}:albumin. Likewise, it was suggested that two tetrapeptides, with one Cu^{2+}-binding site, would inhibit the formation of the Cu^{2+}/DPCH complex at a 1:2 molar ratio of Cu^{2+}:peptide. In support of this, all of the synthetic tetrapeptides commonly exhibited an almost full inhibition of the Cu^{2+}/DPCH complex formation at a 1:2 molar ratio of Cu^{2+}:peptide (**Figure 4B**). These results may corroborate the idea that at least two Cu^{2+}-binding sites are required for the formation of the Cu^{2+}:ligand complex, irrespective of the provider of Cu^{2+}-binding sites, either peptide or protein. Furthermore, the digestion of albumin with trypsin, leading to the generation of smaller peptides, did not affect the inhibitory effect of albumin on Cu^{2+}/DCPH complex formation (**Figure 4C**), suggesting that the N-terminal sequence responsible for Cu^{2+} binding was maintained during the trypsin digestion without losing Cu^{2+}-binding capacity. Meanwhile, the chymotrypsin treatment tended to decrease the inhibitory activity of rice albumin. The difference of Cu^{2+}-binding capacity between chymotrypsin digest and trypsin digest might be due to the different cleavage site. This might be supported by the previous report (*18*) that the antioxidant action of the peptides containing N-terminal sequence of serum albumin seemed to differ according to the size of peptides. Furthermore, the treatment of crude albumin with trypsin or chymotrypsin enhanced the Cu^{2+}-binding capacity (unpublished data), alluding that rice albumin in the protein mixture could exert a Cu^{2+}-removing action in intestinal system.

Taken all together, present data indicate that rice albumin may exert a protective action against Cu^{2+}-catalyzed oxidations in intestinal environment. Such an action of rice albumin is possibly explained by the selective removal of copper ions, due to the presence of N-terminal DHHQ sequence. Additionally, it can exert a HOCl-scavenging effect, probably ascribed to the direct interaction between rice albumin and HOCl. In comparison, rice albumin was less effective than serum albumin in inhibiting Cu^{2+}/DCPH complex formation, which might be due to the fewer number of Cu^{2+}-binding sites in rice albumin, as compared to serum albumin. Nonetheless, rice albumin was as effective as serum albumin in preventing against Cu^{2+}-induced oxidation of LDL, suggesting that one Cu^{2+}-binding site of albumin might be sufficient in preventing against Cu^{2+}-induced oxidation of LDL, where apolipoprotein B already provides an additional Cu^{2+}-binding site (*39*, *40*). From these data, it is proposed that rice albumin as a Cu^{2+} chelator may contribute to the reduction of available free copper ions or exert a preventive action against Cu^{2+}-induced oxidation in intestinal system, probably adding up to the usefulness of rice albumin. Additionally, the Cu^{2+} binding by rice albumin may alter the allergenicity or metabolism of albumin, which needs further studies.

LITERATURE CITED

(1) Harman, D. Role of antioxidant nutrients in aging: Overview. *Age* **1995**, *18*, 51−62.
(2) Ames, B. N. Dietary carcinogens and anticarcinogens: Oxygen radicals and degenerative diseases. *Science* **1983**, *221*, 1256−1264.
(3) Gey, K. F. The antioxidant hypothesis of cardiovascular disease: Epidemiology and mechanisms. *Biochem. Soc. Trans.* **1990**, *18*, 1041−1045.
(4) Yen, G. C.; Chuang, D. Y. Antioxidant properties of water extracts from *Cassia tora* L. in relation to the degree of roasting. *J. Agric. Food Chem.* **2000**, *48*, 2760−2765.

(5) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152−159.
(6) Prior, R. L. Fruits and vegetables in the prevention of cellular oxidative damage. *Am. J. Clin. Nutr.* **2003**, *78* (3 Suppl.), 570S−578S.
(7) Rose, P.; Ong, C. N.; Whiteman, M. Protective effects of Asian green vegetables against oxidant induced cytotoxicity. *World J. Gastroenterol.* **2005**, *11*, 7607−7614.
(8) Clifford, M. N. Diet-derived phenols in plasma and tissues and their implications for health. *Planta Med.* **2004**, *70*, 1103−1114.
(9) Hou, W. C.; Chen, Y. C.; Chen, H. J.; Lin, Y. H.; Yang, L. L.; Lee, M. H. Antioxidant activities of trypsin inhibitor, a 33 kDa root storage protein of sweet potato [*Ipomoea batatas* (L.) Lam cv. Tainong 57]. *J. Agric. Food Chem.* **2001**, *49*, 2978−2981.
(10) Liu, Y. W.; Han, C. H.; Lee, M. H.; Hsu, F. L.; Hou, W. C. Patatin, the tuber storage protein of potato (*Solanum tuberosum* L.), exhibits antioxidant activity in vitro. *J. Agric. Food Chem.* **2003**, *51*, 4389−4393.
(11) Hou, W. C.; Lee, M. H.; Chen, H. J.; Liang, W. L.; Han, C. H.; Liu, Y. W.; Lin, Y. H. Antioxidant activities of dioscorin, the storage protein of yam (*Dioscorea batatas* Decne) tuber. *J. Agric. Food Chem.* **2001**, *49*, 4956−4960.
(12) Nakase, M.; Adachi, T.; Urisu, A.; Miyashita, T.; Alvarez, A. M.; Nagasaka, S.; Aoki, N.; Nakamura, R.; Matsuda, T. Rice (*Oryzae sativa* L.) α-amylase inhibitors of 14−16 kDa are potential allergens and products of a multigene family. *J. Agric. Food Chem.* **1996**, *44*, 2624−2628.
(13) Matsuda, T.; Sugiyama, M.; Nakamura, R. Purification and properties of an allergenic protein in rice grain. *Agric. Biol. Chem.* **1998**, *52*, 1465−1470.
(14) Houston, D. F.; Iwasaki, T.; Mohammad, A.; Chen, L. Radical distribution of protein by solubility classes in milled rice Kernel. *J. Agric. Food Chem.* **1968**, *5*, 720−724.
(15) Emerson, T. E., Jr. Unique features of albumin: A brief review. *Crit. Care Med.* **1989**, *17* (7), 690−694.
(16) Rodriguez de Turco, E. B.; Belayev, L.; Liu, Y.; Busto, R.; Parkins, N.; Bazan, N. G.; Ginsberg, M. D. Systemic fatty acid responses to transient focal cerebral ischemia: Influence of neuroprotectant therapy with human albumin. *J. Neurochem.* **2002**, *83*, 515−524.
(17) Bar-Or, D.; Curtis, G.; Rao, N.; Bampos, N.; Lau, E. Characterization of the CO(2+) and Ni(2+) binding amino-acid residues of the N-terminus of human albumin: An insight into the mechanism of a new assay for myocardial ischemia. *Eur. J. Biochem.* **2001**, *268*, 42−47.
(18) Bar-Or, D.; Thomas, G. W.; Rael, L. T.; Lau, E. P.; Winkler, J. V. Asp-Ala-His-Lys (DAHK) inhibits copper-induced oxidative DNA double strand breaks and telomere shortening. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 356−360.
(19) Bar-Or, D.; Rael, L. T.; Lau, E. P.; Rao, N. K.; Thomas, G. W.; Winkler, J. V.; Yukl, R. L.; Kingston, R. G.; Curtis, C. G. An analog of the human albumin N-terminus (Asp-Ala-His-Lys) prevents formation of copper-induced reactive oxygen species. *Biochem. Biophys. Res. Commun.* **2001**, *284*, 856−862.
(20) Nguyen, S. D.; Jeong, T. S.; Kim, M. R.; Sok, D. E. Broad-spectrum antioxidant peptides derived from His residue-containing sequences present in human paraoxonase 1. *Free Radical Res.* **2006**, *40* (4), 349−358.
(21) Lowry, O. H.; Rosenbrough, G. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(22) Bollag, D. M.; Michael, D. R.; Stuart, J. E. *Protein Methods*, 2nd ed.; Wiley-Liss: New York, 1996.
(23) Laemmli, U. K. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(24) Schumaker, V. N.; Puppione, D. L. Sequential flotation ultracentrifugation. *Methods Enzymol.* **1986**, *128*, 155−170.
(25) Morel, D. W.; Hessler, J. R.; Chisolm, G. M. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J. Lipid Res.* **1983**, *8*, 1070−1076.
(26) Yan, L. J.; Droy-Lefaix, T.; Packer, L. Ginkgo biloba extract (Egb 761) protects human low-density lipoproteins against oxidative modification mediated by copper. *Biochem. Biophys. Res. Commun.* **1995**, *212*, 360−366.
(27) Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351−358.
(28) Lass, A.; Witting, P.; Stocker, R.; Esterbauer, H. Inhibition of copper- and peroxyl radical-induced LDL lipid oxidation by ebselen: Antioxidant actions in addition to hydroperoxide reducing activity. *Biochim. Biophys. Acta* **1996**, *1303*, 111−118.
(29) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: A simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215−219.
(30) Ellman, G. L.; Courtney, K. D.; Andres, V. J.; Feather-stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **1961**, *7*, 88−95.
(31) Jeong, T. S.; Kim, J. R.; Kim, K. S.; Cho, K. H.; Bae, K. H.; Lee, W. S. Inhibitory effects of multi-substituted benzylidenethiazolidine-2,4-diones on LDL oxidation. *Bioorg. Med. Chem.* **2004**, *12* (15), 4017−4023.
(32) Turkington, R. W.; Tracy, F. M. Spectrophotometric determination of ultramicro amounts of copper with 1,5-diphenylcarbohydrazide. *Anal. Chem.* **1958**, *30*, 1699−1701.
(33) Padhye, V. W.; Salunkhe, D. K. Extraction and characterization of rice proteins. *Cereal Chem.* **1979**, *56*, 389−393.
(34) Hidehiko, I.; Takahiro, A.; Noboru, F.; Tsukasa, M.; Rjo, N.; Kunisuke, T.; Atsuo, U.; Yoshikazu, K. Nucleotide sequence of a cDNA clone encoding a major allergenic protein in rice seeds. *FEBS* **1992**, *302* (3), 213−216.
(35) Adachi, T.; Izumi, H.; Yamada, T.; Tanaka, K.; Takeuchi, S.; Nakamura, R.; Matsuda, T. Gene structure and expression of rice allergenic proteins belonging to the α-amylase/trypsin inhibitor family. *Plant Mol. Biol.* **1993**, *21*, 239−248.
(36) Den Hartog, G. J.; Vegt, E.; Van, der Vijgh, W. J.; Haenen, G. R.; Bast, A. Hypochlorous acid is a potent inhibitor of acetylcholinesterase. *Toxicol. Appl. Pharmacol.* **2002**, *181*, 228−232.
(37) Steinbrecher, U. P. Oxidation of human low-density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **1987**, *262*, 3603−3608.
(38) Burkitt, M. J. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: Roles of lipid hydroperoxides, alpha-tocopherol, thiols and ceruloplasmin. *Arch. Biochem. Biophys.* **2001**, *394*, 117−135.
(39) Wagner, P.; Heinecke, J. W. Copper ions promote peroxidation of low density lipoprotein lipid by binding to histidine residues of apolipoprotein B100, but they are reduced at other sites on LDL. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 3338−3346.
(40) Alexander, R.; Rebecca, A. P.; David, S. L. Measurement of copper-binding sites on low density lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* **2001**, *21*, 594−602.

Received for review August 19, 2006. Revised manuscript December 22, 2006. Accepted January 4, 2007. This work was financially supported by the Korean Research Foundation (KRF-2005-042-C20050), Korea.

JF062387G