DOI:10.1021/jf100106e

JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY
ARTICLE

# Effects of Chlorogenic Acid and Bovine Serum Albumin on the Oxidative Stability of Low Density Lipoproteins *in Vitro*

MICHAEL H. GORDON* AND KARL WISHART

Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences,
University of Reading, Whiteknights, PO Box 226, Reading RG6 6AP, U.K.

The ability of chlorogenic acid to inhibit oxidation of human low-density lipoprotein (LDL) was studied by *in vitro* copper-induced LDL oxidation. The effect of chlorogenic acid on the lag time before LDL oxidation increased in a dose dependent manner by up to 176% of the control value when added at concentrations of 0.25−1.0 $\mu$M. Dose dependent increases in lag time of LDL oxidation were also observed, but at much higher concentrations, when chlorogenic acid was incubated with LDL (up to 29.7% increase in lag phase for 10 $\mu$M chlorogenic acid) or plasma (up to 16.6% increase in lag phase for 200 $\mu$M chlorogenic acid) prior to isolation of LDL, and this indicated that chlorogenic acid was able to bind, at least weakly, to LDL. Bovine serum albumin (BSA) increased the oxidative stability of LDL in the presence of chlorogenic acid. Fluorescence spectroscopy showed that chlorogenic acid binds to BSA with a binding constant of $3.88 \times 10^4$ $M^{-1}$. BSA increased the antioxidant effect of chlorogenic acid, and this was attributed to copper ions binding to BSA, thereby reducing the amount of copper available for inducing lipid peroxidation.

**KEYWORDS: Antioxidant; bovine serum albumin; chlorogenic acid; low density lipoprotein; oxidation**

## INTRODUCTION

Oxidized low-density lipoprotein (LDL) has been recognized as a risk factor for cardiovascular disease (CVD) due to its role in the development of atherosclerosis (*1*). Oxidation processes promote a change in LDL particle structure, and oxidized LDL is taken up by the scavenger receptors of macrophages instead of the normal LDL receptors (*1*). This pathway is not downregulated and therefore causes an accumulation of cholesterol derived from LDL particles which eventually leads to the formation of cytosolic droplets and ultimately foam cells (*2*). Oxidized LDL particles also trigger the expression of adhesion molecules, growth factors and cytokines, which are important in the progression of atherosclerosis (*3*).

*In vivo* measurement of LDL oxidation is difficult and has led to the development of a variety of *in vitro* models in order to assess the susceptibility of LDL to oxidation. One of the most widely used techniques measures the resistance of LDL particles to $Cu^{2+}$-induced oxidation. During the lag phase, the lipid peroxidation process is limited due to LDL-associated natural antioxidants such as vitamin E. After all LDL-associated antioxidants have been oxidized, there is a rapid development of conjugated dienes due to lipid peroxidation during the propagation phase (*4*).

Dietary antioxidants have been shown to inhibit oxidative modification of LDL. A number of *in vitro* studies have demonstrated that both water-soluble (e.g., vitamin C) and lipid-soluble (e.g., vitamin E) antioxidants, as well as a variety of phenolic compounds (e.g., catechin, quercetin and myricetin), can inhibit copper-induced LDL oxidation (*5*−*7*). One such phenolic compound is chlorogenic acid, which is present in coffee and in the leaves and fruits of many plants. Chlorogenic acid is also referred to as 5-*O*-caffeoylquinic acid and is an ester of caffeic acid and quinic acid.

Studies investigating the effect of coffee consumption on oxidative susceptibility of LDL and on serum lipid levels in humans have shown that some constituents of coffee, including chlorogenic acid and caffeic acid, could help protect against oxidative modification of LDL. Significant decreases in susceptibility of LDL to oxidation have been observed upon consumption of coffee. It was unclear which compounds were responsible for this increase in LDL stability, but the phenolic components of coffee were identified as the probable cause (*8*). It is known that chlorogenic acid is partly hydrolyzed to caffeic acid in the stomach, but a study found that chlorogenic acid was readily absorbed by humans, resulting in maximum plasma concentrations of 5.9 ± 4.2 $\mu$M (*9*).

Human serum albumin (HSA) is the most abundant protein in human blood plasma, and it shares 76% sequence homology with bovine serum albumin (BSA) (*10*). It has been suggested that BSA inhibits copper-induced LDL oxidation by binding copper ions (*5*), and it also binds a number of phenolic compounds which may affect their ability to act as antioxidants (*11*, *12*). Plasma levels of HSA are in the region of 0.6 mM, so it is clear that HSA is present at relatively high concentrations in plasma compared to phenolic acids such as chlorogenic acid (*9*).

In this study, the antioxidant properties of one of the major phenolic components in coffee, chlorogenic acid, were investigated by its effect on LDL oxidation. Since albumin is the most abundant protein in blood plasma, we attempted to simulate *in vivo* conditions, by investigating the simultaneous effects of BSA and chlorogenic acid on the oxidative modification of LDL. In order to help elucidate

*Corresponding author. Phone: +44 118 3786723. Fax: +44 118 9310 080. E-mail: m.h.gordon@reading.ac.uk.


Published on Web 04/13/2010
© 2010 American Chemical Society

mechanisms of action, the interactions of BSA and chlorogenic acid were also investigated by fluorescence spectroscopy.

## MATERIALS AND METHODS

**Reagents and Apparatus.** Chlorogenic acid (≥95%), BSA (≥96%), copper sulfate (≥99%), phosphate buffered saline (PBS) and all other reagents were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, Dorset, U.K.). Vivaspin 6 centrifugal concentrators were obtained from Fisher Scientific UK Ltd. (Loughborough, U.K.) and PD-10 desalting columns were obtained from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, U.K.). Ultracentrifugation was carried out with a Beckman Coulter Optima L-90K ultracentrifuge with a 65.2 near vertical rotor (Beckman Coulter UK Ltd., High Wycombe, U.K.). Absorbance measurements for the Lowry Assay were carried out with a Tecan platereader (Tecan Ltd., Weymouth, Dorset, U.K.) connected to a computer running Magellan 5 software. Absorbance measurements for LDL oxidation experiments were performed using a Perkin-Elmer Lambda Bio 20 UV/vis spectrophotometer (PerkinElmer UK Ltd., Seer Green, U.K.). Fluorescence was measured with a Varian Cary Eclipse Fluorescence Spectrophotometer (Varian Ltd., Oxford, U.K.).

**Isolation and Preparation of LDL.** LDL was isolated from human plasma by ultracentrifugation using the method of Leigh-Firbank et al. (*13*). LDL was resuspended in PBS to give a final protein concentration of 100 $\mu$g/mL. LDL was stored at −80 °C in a nitrogen atmosphere.

This LDL isolation process was repeated for experiments in which LDL suspensions were preincubated with chlorogenic acid (0−10 $\mu$M) or aliquots of plasma were preincubated with chlorogenic acid (0−200 $\mu$M).

**Determination of Protein Content of LDL.** A modified Lowry assay was used to determine the protein content of LDL (*14*). Several protein standards of different concentrations (0, 25, 50, 75, 100, 150, 200, and 250 $\mu$g/mL) were prepared by diluting a 400 $\mu$g/mL BSA stock solution. LDL samples were diluted 1:25 with distilled water. Triplicate aliquots (40 $\mu$L) of protein standards and LDL sample were transferred to a 96-well microplate. Lowry A solution (40 $\mu$L; 0.94 M $Na_2CO_3$, 0.5 M NaOH, 7.09 mM $KNaC_4H_4O_6 \cdot 4H_2O$ and $CuSO_4 \cdot 5H_2O$) was added to each well. After 10 min, Lowry N solution (160 $\mu$L; 1 mL of Folin and Ciocalteu phenol reagent in 14 mL of distilled water) was added to each well and mixed immediately before incubating the wells in a 55 °C water bath for 5 min. Absorbance was then measured at 650 nm using a Tecan platereader.

**Measurement of LDL Oxidation.** LDL oxidation was measured by monitoring the formation of conjugated dienes, which are breakdown products of lipid peroxidation. LDL in PBS dispersion was diluted to a concentration of 50 $\mu$g of protein/mL. In addition to a control containing only LDL and PBS, 6 different samples were prepared with a range of concentrations of chlorogenic acid (CGA) (0.25−1 $\mu$M CGA) and BSA (0% or 4%, 0.6 mM, $4 \times 10^4$ $\mu$g/mL). After incubating for 1 h at 37 °C, $CuSO_4$ (0.1 mM) was added to the dispersions to yield a total $CuSO_4$ concentration of 5 $\mu$M. Absorbance measurements at 234 nm were started immediately after addition of $CuSO_4$, and recorded every 2 min for 3 h.

In a separate experiment, samples of LDL were incubated with higher concentrations of chlorogenic acid (1.0−10.0 $\mu$M CGA and 0% or 4% BSA) at 37 °C for 1 h. Excess free chlorogenic acid was then removed with a PD-10 desalting column. $CuSO_4$ was then added to the samples, and the absorbance was measured. Additional LDL oxidation experiments were performed on LDL incubated with BSA, as well as on LDL isolated from plasma preincubated with different concentrations of chlorogenic acid (50, 100, and 200 $\mu$M).

The lag time was calculated by determining the intercept of the tangents to the lag and propagation phases.

**Fluorescence Quenching.** Fluorescence quenching measurements were carried out in triplicate for solutions containing 0.3 $\mu$M BSA and varying concentrations of chlorogenic acid (0, 1, 5, 10, 15, 20 $\mu$M) in PBS at 37 °C. The excitation wavelength was set at 282 nm and emission spectra were recorded from 290 to 500 nm with excitation and emission slit widths of 10 nm. Fluorescence intensity data was evaluated as described in previous studies (*15*, *16*). The data was fitted to the Stern−Volmer equation (eq 1)

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \qquad (1)$$

where $F_0$ is the fluorescence intensity before addition of chlorogenic acid and $F$ is the fluorescence intensity after addition of chlorogenic acid, $\tau_0$ is the lifetime of the fluorophore in the absence of chlorogenic acid (for BSA $\tau_0 = 5$ ns (*15*)), [Q] is the concentration of chlorogenic acid and $K_{SV}$ is the Stern−Volmer quenching constant. A linear Stern−Volmer plot indicates that the fluorophores in the protein are all equally accessible by the quencher, and that quenching occurs via either a static or dynamic mechanism. However when the effect of quenching is large, the Stern−Volmer plot has an upward curvature at large quencher concentrations. This deviation from the linear Stern−Volmer plot is often attributed to the quencher acting by both mechanisms (dynamic and static) simultaneously or in some cases can be attributed to the presence of a so-called sphere of action. The sphere of action model describes a sphere of volume around a fluorophore in which a quencher will act upon the fluorophore.

The binding constant, $K_A$, for the BSA−chlorogenic acid complex was calculated from eq 2 (*17*):

$$\frac{F_0}{F_0 - F} = 1 + \frac{1}{K_A}\frac{1}{[Q]} \qquad (2)$$

**Statistics.** Triplicate measurements were carried out for all experiments, and statistical analysis was carried out with the Origin Pro Software (OriginLab Corporation, Northampton, MA). Analysis of variance (ANOVA) followed by a Tukey post-hoc test was used to compare LDL oxidation lag phase times. Results with $P \leq 0.05$ were regarded as statistically significant.

**Table 1.** LDL Oxidation Lag Time for Samples Incubated with Various Concentrations of Chlorogenic Acid and Bovine Serum Albumin under Different Experimental Conditions: (**a**) Incubation with LDL without Removal of Chlorogenic Acid Prior to Oxidation; (**b**) Incubation of LDL with Chlorogenic Acid Removed Prior to Oxidation; (**c**) Plasma Incubated with Chlorogenic Acid Prior to Isolation of LDL and Oxidation; (**d**) LDL with BSA[a]

| sample | lag period (min) |
|---|---|
| (**a**) Without Removal of Chlorogenic Acid | |
| control | 41.77 ± 1.94 a |
| 0.25 $\mu$M CGA | 56.43 ± 2.67 b |
| 0.50 $\mu$M CGA | 71.26 ± 2.35 c |
| 1.00 $\mu$M CGA | 115.27 ± 3.61 d |
| 0.25 $\mu$M CGA + 4% BSA | 54.83 ± 2.53 b |
| 0.50 $\mu$M CGA + 4% BSA | 80.28 ± 2.65 e |
| 1.00 $\mu$M CGA + 4% BSA | 143.47 ± 7.03 f |
| (**b**) With Removal of Chlorogenic Acid | |
| control | 37.72 ± 2.29 a |
| 1.00 $\mu$M CGA | 42.61 ± 1.75 b |
| 5.00 $\mu$M CGA | 47.46 ± 0.93 c |
| 10.00 $\mu$M CGA | 48.91 ± 1.14 c |
| 1.00 $\mu$M CGA + 4% BSA | 47.57 ± 1.57 c |
| 5.00 $\mu$M CGA + 4% BSA | 52.90 ± 1.30 d |
| 10.00 $\mu$M CGA + 4% BSA | 64.02 ± 0.74 e |
| (**c**) Plasma Incubated with Chlorogenic Acid | |
| control | 52.93 ± 1.29 a |
| 50.0 $\mu$M CGA | 54.99 ± 0.66 b |
| 100.0 $\mu$M CGA | 57.53 ± 0.80 c |
| 200.0 $\mu$M CGA | 61.74 ± 1.21 d |
| (**d**) LDL with BSA | |
| control | 26.88 ± 3.77 a |
| 4% BSA | 27.44 ± 3.60 a |

[a] Values were calculated by determining the intercept of the tangents to the lag phase and propagation phase. All values are mean ± SEM. Values obtained under the same experimental conditions with different letters are significantly different ($P < 0.05$).

## RESULTS

**LDL Oxidation.** Oxidation of LDL was induced by the addition of $Cu^{2+}$. The lag times corresponding to the induction period for LDL oxidation are summarized in **Table 1**. An increase in

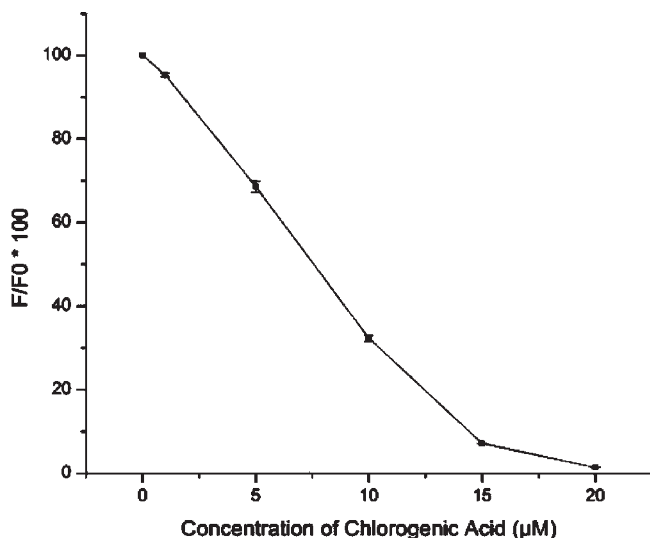


**Figure 1.** Fluorescence quenching of BSA (0.3 $\mu$M) shown as extinction of BSA fluorescence ($F/F_0 \times 100$) against chlorogenic acid concentration. The fluorescence emission intensity was recorded at $\lambda_{ex}$ = 282 nm, and the $\lambda_{em}$ maximum occurred at 344 nm.

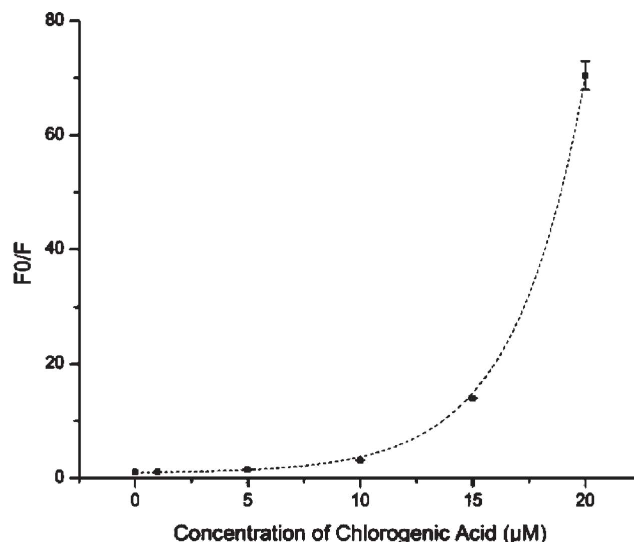


**Figure 2.** Stern−Volmer plot describing quenching of BSA (0.3 $\mu$M) in the presence of chlorogenic acid at different concentrations (0, 1, 5, 10, 15, 20 $\mu$M). Equation: $y = 0.11525\ e^{x/3.12381}$. The fluorescence emission intensity was recorded at $\lambda_{ex}$ = 282 nm, and the $\lambda_{em}$ maximum occurred at 344 nm.

chlorogenic acid concentration significantly increased the lag time. This concentration dependent effect was achieved when LDL was incubated with lower concentrations of chlorogenic acid (0.25, 0.5, 1.0 $\mu$M) and oxidation was performed without removal of free chlorogenic acid. A concentration dependent increase in lag time was also observed when LDL was incubated with higher concentrations of chlorogenic acid (1.0, 5.0, 10.0 $\mu$M) after which free chlorogenic acid was removed from the samples before oxidation, although the magnitude of the increase was modest with only a 29.7% increase for 10 $\mu$M chlorogenic acid. The latter case did not show a significant increase in lag time for a chlorogenic acid concentration above 5 $\mu$M when BSA was not present. Lag times were longer when free chlorogenic acid was present during oxidation compared to when free chlorogenic acid was removed prior to oxidation, indicating that free chlorogenic acid plays an important role in inhibiting LDL oxidation. A modest increase in lag time before LDL oxidation was also seen when LDL was isolated from blood plasma that was preincubated with chlorogenic acid, but higher concentrations (50−200 $\mu$M) were required, and the effect only reached 16.6% increase in lag time for 200 $\mu$M chlorogenic acid .

The addition of 4% BSA (0.6 mM) to the samples without removal of free chlorogenic acid, as well as to the samples with removal of free chlorogenic acid, also prolonged the lag time before LDL oxidation. However, this effect was only significant at higher chlorogenic acid concentrations ($\geq$0.5 $\mu$M), as shown in **Table 1**.

**Fluorescence Quenching.** The interaction of BSA with chlorogenic acid was studied by fluorescence spectroscopy. The fluorescence spectra were recorded for 0.3 $\mu$M BSA with varying concentrations of chlorogenic acid (0−20 $\mu$M). A decrease in fluorescence intensity was observed with an increase in chlorogenic acid concentration indicating an interaction of chlorogenic acid with BSA. From the fluorescence spectra a plot was constructed showing the effect of chlorogenic acid concentration on BSA fluorescence (**Figure 1**). An almost linear reduction in fluorescence was observed up to 15 $\mu$M chlorogenic acid, and BSA fluorescence was close to zero by 20 $\mu$M, indicating a strong interaction of chlorogenic acid with BSA. The Stern−Volmer plot for fluorescence quenching of BSA by chlorogenic acid is shown in **Figure 2**. The upward curvature indicates that chlorogenic acid quenches BSA either by static and dynamic mechanisms simultaneously or by the sphere of action model. The binding constant, $K_A$, can be calculated to be $3.88 \times 10^4$ M $^{-1}$ from the gradient of the plot of $F_0/(F_0 - F)$ against the reciprocal of the chlorogenic acid concentration (**Figure 3**). This compares with the value of $4.37 \times 10^4$ M $^{-1}$ for the binding constant of the chlorogenic acid−human serum albumin complex (*17*).

## DISCUSSION

This study showed that chlorogenic acid increased the resistance of LDL to oxidative modification. It also showed that BSA is capable of enhancing the effect of chlorogenic acid in decreasing the susceptibility of LDL to $Cu^{2+}$-induced oxidation.

*In vitro* $Cu^{2+}$-catalyzed LDL oxidation showed that chlorogenic acid exerted its antioxidant effects in a concentration dependent manner (**Table 1**). The fact that chlorogenic acid had a modest antioxidant effect even when a desalting cartridge, which gave a good separation between phenolic acids and proteins, was used to remove free chlorogenic acid indicates that chlorogenic acid bound to LDL particles, as has been observed for other phenolic compounds (*7*, *18*) Bound chlorogenic acid contributed to the inhibitory effect toward LDL oxidation, either by a direct effect, by release of free chlorogenic acid from a weak complex with LDL, or by inhibiting the binding of copper to the LDL particles. However, the effect of free chlorogenic acid was greater than that of chlorogenic acid added prior to isolation of LDL (**Table 1**) where much higher concentrations were needed, and the antioxidant effect was smaller. The concentration of chlorogenic acid bound to LDL was probably small.

The results obtained are consistent with previous studies which also reported a decreased susceptibility of LDL toward oxidation in the presence of chlorogenic acid (*19*, *20*). The activity of chlorogenic acid as a potent antioxidant is due to various structural features, including the presence of ortho-hydroxyl groups attached to the aromatic ring.

The mechanisms by which chlorogenic acid inhibits $Cu^{2+}$-catalyzed LDL oxidation have not been completely elucidated, although a number of mechanisms have been proposed which include free radical scavenging, transient chelation of $Cu^{2+}$ ions, and steric blockage of $Cu^{2+}$ binding sites on LDL (*21*−*24*). One

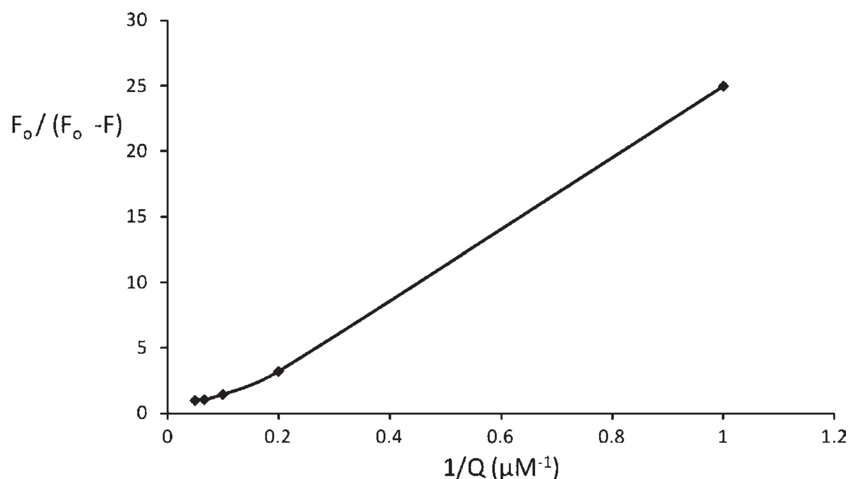


**Figure 3.** Double reciprocal curve of protein fluorescence quenching in the presence of chlorogenic acid (concentration Q). The data fits the equation $y = 25.794x - 0.952$. $R^2 = 0.996$.

mechanism suggests chlorogenic acid scavenges free radicals thereby enabling it to inhibit the lipid peroxidation process by reducing alkoxyl or peroxyl radicals to alkoxides or hydroperoxides (*21*). The chelation of $Cu^{2+}$ ions by compounds containing ortho-dihydroxyl groups was demonstrated by Nardini et al. who proposed the formation of a $Cu^{2+}$−caffeic acid complex, thereby restricting the ability of $Cu^{2+}$ to promote lipid peroxidation (*22*). The structural similarity of caffeic acid and chlorogenic acid would suggest that chlorogenic acid acts by the same mechanism, as confirmed in a separate study (*23*). Roland et al. proposed a mechanism by which $Cu^{2+}$ binding to LDL particles is inhibited (*24*). Their data showed that LDL possesses 38 different $Cu^{2+}$ binding sites. At the same time phenolic compounds, including myricetin, quercetin and catechin, were shown to significantly inhibit $Cu^{2+}$ binding to LDL, thereby limiting the ability of $Cu^{2+}$ to induce oxidation. It is likely that chlorogenic acid acts by the same mechanism, but this has yet to be confirmed.

The possibility of chlorogenic acid being oxidized to quinones, which react with amine side chains of lysine residues in the LDL particle, needs to be considered, since the oxidation potential of chlorogenic acid is low at +0.23 V (*25*). However, the chlorogenic acid concentration was $< 1$ $\mu$M, with relatively high concentrations of lysine in LDL (8.2 mol %) (*26*). Since chlorogenic acid has strong antioxidant activity when added to the LDL suspension prior to oxidation, it can be deduced that reaction of chlorogenic acid by oxidation and covalent binding to LDL is slow compared to the time scale of the experiment. Cu(II) will remain as the main copper ion in these experiments since it is present at 5 $\mu$M, compared to concentrations of chlorogenic acid, which are not expected to be higher than 1 $\mu$M in any of the experiments, since free chlorogenic acid is removed if added prior to isolation of LDL.

Increased transient chelation of $Cu^{2+}$ ions, increased radical scavenging and blockage of more copper binding sites to LDL by chlorogenic acid may explain the increased lag times for LDL oxidation experiments in the presence of free chlorogenic acid (**Table 1a**) compared to experiments with removal of free chlorogenic acid before oxidation (**Table 1b**).

One of the main aims of this study was to determine the simultaneous effect of BSA and chlorogenic acid on LDL oxidation. This aim increases the nutritional relevance of the *in vitro* experiment, since HSA, the human homologue of BSA, is the most abundant protein in blood plasma. Results summarized in **Table 1** clearly show that, in the presence of higher ($\geq$0.5 $\mu$M) concentrations of chlorogenic acid, BSA (4%, 0.6 mM) has a significant protective effect against LDL oxidation. Also, the finding that, even at low (0.25 $\mu$M) concentrations of chlorogenic acid, BSA did not reduce the lag time shows that BSA does not prevent the antioxidant effects of chlorogenic acid, despite the fact that chlorogenic acid is capable of binding to BSA, as shown by fluorescence spectroscopy. The mechanism by which BSA may exert its inhibitory effect toward LDL oxidation is by binding to $Cu^{2+}$ ions as described in previous studies (*27*−*29*). However, when LDL was incubated with 4% BSA without chlorogenic acid no significant protective effect toward LDL oxidation was observed (**Table 1d**). This shows that BSA is not an effective radical scavenger, and it is consistent with previous findings which suggested that the effect of BSA is dependent on the concentration of BSA relative to that of copper (*5*). According to these findings, 4% BSA (0.6 mM) is not sufficient on its own to inhibit LDL oxidation induced by 5 $\mu$M $Cu^{2+}$ ions, although this yields a BSA to copper molar concentration ratio of 125:1, and the binding constant for copper binding to BSA has been reported as $2.78 \times 10^{-2}$ (*29*). However, when 4% BSA and $\geq$0.5 $\mu$M chlorogenic acid are both present, LDL oxidation is inhibited due to the combined effects of BSA binding $Cu^{2+}$, and the free radical scavenging actions of chlorogenic acid, which lead to increased lag times for samples containing BSA and chlorogenic acid compared to samples containing just chlorogenic acid. This indicates the important role of proteins in reducing free copper concentrations, which allows phenolic antioxidants to be active antioxidants even at low concentrations *in vivo*. Other proteins in plasma such as ceruloplasmin and lactoferrin, the copper- and iron-carrying proteins, respectively, play a major role in maintaining very low concentrations of unbound copper and iron ions in the circulation. However, ceruloplasmin has still been shown to catalyze LDL oxidation (*30*, *31*).

In a further attempt to simulate *in vivo* conditions, plasma was preincubated for 1 h with different concentrations of chlorogenic acid (0−200 $\mu$M), after which LDL was isolated. Results showed a significant increase in resistance of LDL to oxidation with increasing concentrations of chlorogenic acid (**Table 1c**). This confirms that chlorogenic acid is able to bind to LDL *in vitro* and exert its antioxidant effects. The importance of this was established in a previous study which showed that antioxidant effects of compounds varied considerably when comparing *ex vivo* and *in vitro* results (*32*). This variation was attributed to the different protein binding affinities of phenolic compounds, which resulted in different binding affinities to LDL particles. Phenolic compounds with strong binding affinities to proteins showed the

largest correlation between antioxidant effects in *ex vivo* and *in vitro* experiments. It was therefore hypothesized that not only the antioxidant activity of compounds was important but also their ability to bind to LDL particles.

The binding of chlorogenic acid to BSA was confirmed by fluorescence quenching experiments, and this suggests that chlorogenic acid can also bind to tryptophan residues of LDL particles. The ability of chlorogenic acid to bind to LDL particles could in part play a role in the antioxidant mechanism of chlorogenic acid. Giessauf et al. showed that $Cu^{2+}$-induced oxidation of tryptophan residues on apolipoprotein B played an important role in the initiation of LDL oxidation (*33*). Results from fluorescent quenching experiments suggest that binding of chlorogenic acid to tryptophan residues of apolipoprotein B may block $Cu^{2+}$ access to tryptophan residues in LDL, thereby inhibiting LDL oxidation. The binding of chlorogenic acid to human serum albumin is important when considering antioxidant actions of chlorogenic acid *in vivo*.

In conclusion, chlorogenic acid is a potent antioxidant that is capable of inhibiting LDL oxidation by a variety of mechanisms. BSA is capable of enhancing the effects of chlorogenic acid by simultaneously chelating copper ions. Binding of chlorogenic acid to BSA did not impair the antioxidant activity of chlorogenic acid, which was enhanced by the presence of BSA. The results suggest that the antioxidant effects of chlorogenic acid *in vivo* (in the presence of serum albumin) may be stronger than its measured antioxidant effects *in vitro*.

**Supporting Information Available:** Fluorescence spectra for BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

## LITERATURE CITED

(1) Steinberg, D. Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.* **1997**, *272*, 20963–20966.

(2) Hansson, G. K. Mechanism of disease: Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* **2005**, *352*, 1685–1695.

(3) Siekmeier, R.; Steffen, C.; Marz, W. Role of oxidants and antioxidants in atherosclerosis: Results of in vitro and in vivo investigations. *J. Cardiovasc. Pharmacol. Ther.* **2007**, *12*, 265–291.

(4) Esterbauer, H.; Striegl, G.; Puhl, H.; Rothender, M. Continuous montoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67–75.

(5) Schnitzer, E.; Pinchuk, I.; Bor, A.; Fainaru, M.; Lichtenberg, D. The effect of albumin on copper-induced LDL oxidation. *Biochim. Biophys. Acta* **1997**, *1344*, 300–311.

(6) Carr, A. C.; Zhu, B. Z.; Frei, B. Potential antiatherogenic mechanisms of ascorbate (vitamin C) and α-tocopherol (vitamin E). *Circulation* **2000**, *87*, 349–354.

(7) Wu, C. H.; Lin, J. A.; Hsieh, W. C.; Yen, G. C. Low-Density-Lipoprotein (LDL)-Bound Flavonoids Increase the Resistance of LDL to Oxidation and Glycation under Pathophysiological Concentrations of Glucose in Vitro. *J. Agric. Food Chem.* **2009**, *57*, 5058–5064.

(8) Yukawa, G. S.; Mune, M.; Otani, H.; Tone, Y.; Liang, X. M.; Iwahashi, H.; Sakamoto, W. Effects of coffee consumption on oxidative susceptibility of low-density lipoproteins and serum lipid levels in humans. *Biochemistry* (*Moscow*) **2004**, *69*, 70–74.

(9) Farah, A.; Monteiro, M.; Donangelo, C. M.; Lafay, S. Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans. *J. Nutr.* **2008**, *138*, 2309–2315.

(10) Huang, B. X.; Kim, H. Y.; Dass, C. Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. *J. Am. Soc. Mass Specrom.* **2004**, *15*, 1237–1247.

(11) Rawel, H. M.; Frey, S. K.; Meidtner, K.; Kroll, J.; Schweigert, F. J. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.* **2006**, *50*, 705–713.

(12) Rohn, S; Rawel, H. M.; Kroll, J. Antioxidant activity of protein-bound quercetin. *J. Agric. Food Chem.* **2004**, *52*, 4725–4729.

(13) Leigh-Firbank, E. C.; Minihane, A. M.; Leake, D. S.; Wright, J. W.; Murphy, M. C.; Griffin, B. A.; Williams, C. M. Eicosapentanoic acid and docosahexanoic differential associations with lipid responses. *Br. J. Nutr.* **2002**, *87*, 435–445.

(14) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(15) Soares, S.; Mateus, N.; Freitas, V. Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *J. Agric. Food Chem.* **2007**, *55*, 6726–6735.

(16) Johansson, J. S. Binding of the volatile anaesthetic chloroform to albumin demonstrated using tryptophan fluorescence quenching. *J. Biol. Chem.* **1997**, *272*, 17961–17965.

(17) Kang, J.; Liu, Y.; Xie, M.-X.; Li, S.; Jiang, M.; Wang, Y.-D. Interactions of human serum albumin with chlorogenic acid and ferulic acid. *Biochim. Biophys. Acta* **2004**, *1674*, 205–214.

(18) Ivanov, V.; Carr, A. C.; Frei, B. Red wine antioxidants bind to human lipoproteins and protect them from metal ion-dependent and -independent oxidation. *J. Agric. Food Chem.* **2001**, *49*, 4442–4449.

(19) Meyer, A. S.; Donovan, J. L.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro. *J. Agric. Food Chem.* **1998**, *46*, 1783–1787.

(20) Cheng, J. C.; Dai, F.; Zhou, B.; Yang, L.; Liu, Z. L. Antioxidant activity of hydroxycinnamic acid derivatives in human low density lipoprotein: mechanism and structure-activity relationship. *Food Chem.* **2007**, *104*, 132–139.

(21) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152–159.

(22) Nardini, M.; Daquino, M.; Tomassi, G.; Gentili, V.; Difelice, M.; Scaccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541–552.

(23) Kiss, T.; Nagy, G.; Pecsi, M.; Kozlowski, H.; Micera, G.; Erre, L. S. Complexes of 3,4-dihydroxyphenyl derivatives- x copper(II)-complexes of chlorogenic acid and related compounds. *Polyhedron* **1989**, *8*, 2345–2349.

(24) Roland, A.; Patterson, R. A.; Leake, D. S. Measurement of copper-binding sites on low density lipoprotein. *Arterioscler., Thromb., Vasc. Biol.* **2001**, *21*, 594–602.

(25) Born, M.; Carrupt, P. A.; Zini, R.; Bree, F.; Tillement, J. P.; Hostetmann, K.; Testa, B. Electrochemical behaviour and antioxidant activity of some natural polyphenols. *Helv. Chim. Acta* **1996**, *79*, 1147–1158.

(26) Yang, C. Y.; Chen, S. H.; Gianturco, S. H.; Bradley, W. A.; Sparrow, J. T.; Tanimura, M.; Li, W. H.; Sparrow, D. A.; Deloof, H.; Rosseneu, M.; Lee, F. S.; Gu, Z. W.; Gotto, A. M.; Chan, L. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein-B-100. *Nature* **1986**, *323*, 738–742.

(27) Masuoka, J.; Hegenauer, J.; Vandyke, B. R.; Saltman, P. Intrinsic stoichiometric equilibrium constants for the binding of zinc(II) and copper(II) to the high affinity site of serum albumin. *J. Biol. Chem.* **1993**, *268*, 21533–21537.

(28) Zgirski, A.; Frieden, E. A kinetic study on the distribution of Cu(II)-ions between albumin and transferrin. *J. Inorg. Biochem.* **1990**, *39*, 137–148.

(29) Zhang, Y; Akilesh, S; Wilcox, D. E. Isothermal titration calorimetry measurements of Ni(II) and Cu(II) binding to His, GlyGlyHis, HisGlyHis, and bovine serum albumin: A critical evaluation. *Inorg. Chem.* **2000**, *39*, 3057–3064.

(30) Ehrenwald, E.; Chisolm, G. M.; Fox, P. L. Intact human ceruloplasmin oxidatively modifies low density lipoproteins. *J. Clin. Invest.* **1994**, *93*, 1493–1501.

(31) Lamb, D. J.; Leake, D. S. Acidic pH enables ceruloplasmin to catalyse the modification of low density lipoprotein. *FEBS Lett.* **1994**, *338*, 122–126.

(32) Wang, W. Q.; Goodman, M. T. Antioxidant property of dietary phenolic agents in a human LDL-oxidation ex vivo model: interaction of protein binding activity. *Nutr. Res. (N.Y.)* **1999**, *19*, 191–202.
(33) Giessauf, A.; Steiner, E.; Esterbauer, H. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1995**, *1256*, 221–232.

**Received for review January 11, 2010. Revised manuscript received March 29, 2010. Accepted March 30, 2010.**