JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Serotonin Derivatives, Major Safflower (*Carthamus tinctorius* L.) Seed Antioxidants, Inhibit Low-Density Lipoprotein (LDL) Oxidation and Atherosclerosis in Apolipoprotein E-Deficient Mice

NAOTO KOYAMA,*,† KANNA KURIBAYASHI,† TETSUYA SEKI,† KATSUNORI KOBAYASHI,† YASUFUMI FURUHATA,† KATSUYA SUZUKI,† HARUMI ARISAKA,† TAKASHI NAKANO,‡ YUSUKE AMINO,‡ AND KOICHI ISHII†

Research Institute for Health Fundamentals, Ajinomoto Co., Inc., Kawasaki 210-8681, Japan, and Applied Research Department, AminoScience Laboratories, Ajinomoto Co., Inc., Kawasaki 210-8681, Japan

The effects of defatted safflower seed extract and its phenolic constituents, serotonin derivatives, on atherosclerosis were studied. Ethanol−ethyl acetate extract of safflower seeds (SSE) inhibited low-density lipoprotein (LDL) oxidation induced in vitro by an azo-containing free-radical initiator V70 or copper ions. Two serotonin derivatives [*N*-(*p*-coumaroyl)serotonin, CS; *N*-feruloylserotonin, FS] and their glucosides were identified as the major phenolic constituents of the extract. The study with chemically synthesized materials revealed that a majority of the antioxidative activity of SSE was attributable to the aglycones of these two serotonin derivatives. Orally administered CS and FS suppressed $CuSO_4$-induced plasma oxidation ex vivo. Long-term (15 week) dietary supplementation of SSE (1.0 wt %/wt) and synthetic serotonin derivatives (0.2−0.4%) significantly reduced the atherosclerotic lesion area in the aortic sinus of apolipoprotein E-deficient mice (29.2−79.7% reduction). The plasma level of both lipid peroxides and anti-oxidized LDL autoantibody titers decreased concomitantly with the reduction of lesion formation. Serotonin derivatives were detected as both intact and conjugated metabolites in the plasma of C57BL/6J mice fed on 1.0% SSE diet. These findings demonstrate that serotonin derivatives of SSE are absorbed into circulation and attenuate atherosclerotic lesion development possibly because of the inhibition of oxidized LDL formation through their strong antioxidative activity.

KEYWORDS: Safflower; serotonin derivatives; antioxidants; LDL oxidation; apoE-deficient mice

INTRODUCTION

Risk factors such as hyperlipidemia, hypertension, and hyperglycemia are believed to increase oxidative stress in the vascular wall by multiple mechanisms, thereby resulting in upregulation of reactive oxygen species (ROS) production. Proinflammatory cells that infiltrate into the vascular wall secrete large amounts of ROS and oxidative enzymes and lead to oxidative modification of low-density lipoprotein (LDL) and foam cell deposition, the hallmark of early atherogenesis. Thus, attenuation of oxidative stress is one of the major issues in the prevention of initiation and progression of atherosclerosis. Many epidemiological and clinical studies have suggested that antioxidants play a preventive role in atherogenesis; however, many large-scale intervention trials have failed to demonstrate the beneficial effects of antioxidant vitamins. Recently, researchers have expressed a keen interest in the biological activity of plant phenolics as a dietary source of antioxidants. In vivo antiatherogenic activity in conjunction with antioxidative activity has been demonstrated in green tea catechins (*1*), proanthocyanidins (*2*), quercetin glucosides (*3*, *4*), cacao liquor polyphenols (epicatechin and procyanidins) (*5*), and licorice extract (glabridin) (*6*). Despite the wide diversity of phenolics in the plant kingdom, to date, only a limited number of phenolics have been identified as antiatherogenic principals, and in most cases, they are either flavonoids or isoflavonoids. Detailed information on the antiatherogenic properties of plant phenolics from other subclasses will provide a better and rational understanding of the relationship between their antioxidant activity and prophylactic effects on atherosclerosis.

Safflower (*Carthamus tinctorius* L.) seeds have been produced worldwide for a long time period for edible oil production. In addition, they have been used as an herbal medicine in Korea

* To whom correspondence should be addressed: Research Institute for Health Fundamentals, Ajinomoto Co., Inc. 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan. Telephone: +81-44-210-5767. Fax: +81-44-211-7609. E-mail: naoto_koyama@ajinomoto.com.
† Research Institute for Health Fundamentals.
‡ Applied Research Department, AminoScience Laboratories.

10.1021/jf060254p CCC: $33.50
Published on Web 06/14/2006

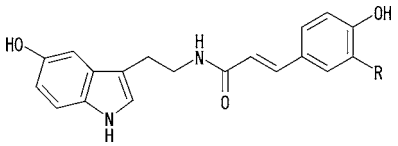

Figure 1. Structure of serotonin derivatives CS, R═H; FS, R═OCH_3.

for the promotion of bone formation and in the treatment of osteoporosis and rheumatism. Serotonin derivatives (**Figure 1**) were identified as the major and unique phenolic constituents of defatted safflower seeds (*7*). These are members of a group of indole hydroxycinnamic acid amides. Serotonin derivatives have been reported to possess strong antioxidative activities in vitro and exert various biological effects on plasma and liver lipid status (*8*), viability and growth of cancer cell lines or fibroblasts (*9*, *10*), ischemia-reperfused Langendorff hearts (*11*), and cellular proinflammatory cytokine or melanin production (*12*, *13*). However, few reports ascribe a particular in vivo effect to these little-known polyphenols.

The purpose of the present study was to investigate whether the extract of defatted safflower seeds and serotonin derivatives exert beneficial effects on atherosclerosis in vitro and in vivo. For this purpose, safflower seed extract and synthetic serotonin derivatives were prepared and their effects on the resistance of LDL to in-vitro-induced oxidation and on aortic lesion development in apoE-deficient mice were analyzed; in addition, biological availability of serotonin derivatives was also examined.

MATERIALS AND METHODS

Defatted safflower seeds were provided by J-Oil Mills, Inc. (Tokyo, Japan). V70 (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), *p*-coumaric acid, Folin-Ciocalteu's phenol reagent, β-glucuronidase (type X-A), and sulfatase (type H5) were purchased from Sigma−Aldrich (St. Louis, MO). Serotonin hydrochloride and *trans*-ferulic acid were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and β-glucosidase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Preparation of Defatted Safflower Seed Extract (SSE) and Analysis of Major Phenolics. A total of 100 g of defatted safflower seeds was extracted twice in 500 mL of 90% (v/v) ethanol for 3 h at 60 °C. The extracted solvent was then concentrated in vacuo and diluted with water to a final volume of 200 mL, followed by *n*-hexane washing (twice). The aqueous layer (100 mL) was then extracted twice with ethyl acetate and vacuum-dried. The term "SSE" in this paper refers to this ethanol−ethyl acetate extract of defatted safflower seeds. Total polyphenol content of the extract was determined by the Folin-Ciocalteu method (*14*).

The extract was analyzed by high-performance liquid chromatography (HPLC) equipped with a photodiode array detector (Alliance; Waters, Milford, MA). The conditions for HPLC were as follows: column, Capcell Pak ODS UG-120 (4.6 × 250 mm, Shiseido Co., Tokyo, Japan); mobile phase, linear gradient of water−acetonitrile (containing 0.1% TFA), 0−35% acetonitrile over 35 min; flow rate, 1 mL/min; detection, 290 nm for CS, FS, CS−monoglucoside, and FS−monoglucoside, 279 nm for 2-hydroxyarctiin; temperature, room temperature. To identify glucosides from the serotonin derivatives, enzymatic hydrolysis of the glucosides was performed with β-glucosidase [1 unit/mL in 0.1% BSA (0.1 mol)$^{-1}$ (L of sodium phosphate buffer)$^{-1}$ at pH 7.0] (24 h, room temperature) and the products were compared with authentic serotonin derivatives by HPLC. 2-Hydroxyarctiin was separated and detected in accordance with the method of Lyon et al. (*15*).

Chemical Synthesis of Serotonin Derivatives. *N-(p-Coumaroyl)-serotonin (CS).* Triethylamine (2.20 mL, 15.78 mmol), 1-hydroxybenzotriazole (2.10 g, 15.54 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (2.97 g, 15.49 mmol) were added to a solution of serotonin hydrochloride (3.00 g, 14.11 mmol) and *p*-coumaric acid (2.32 g, 14.13 mmol) in dimethylformamide (15 mL) and dichloromethane (100 mL). After the mixture was stirred overnight at room temperature, it was concentrated in vacuo. The residue was treated with water (100 mL) and extracted with ethyl acetate (3 × 100 mL). The organic extract was washed successively with 5% citric acid solution (2 × 100 mL), saturated sodium hydrogencarbonate solution (2 × 100 mL), and brine (100 mL). The extract was dried over $MgSO_4$ and concentrated to yield a solid that, upon crystallization from ethyl acetate−ethanol (15:1), yielded 2.87 g of CS (8.89 mmol, 63.0%) as crystals (ESI−MS *m*/*z*: 321.11 $[M - H]^-$).

N-Feruloylserotonin (FS). FS was synthesized in 65.6% yield from serotonin hydrochloride and *trans*-ferulic acid in a similar manner as described above, with the exception that the crystallization was performed using methanol-chloroform (1:15) (ESI−MS *m*/*z*: 351.14 $[M - H]^-$).

The ^{1}H NMR spectra of these compounds were identical to those reported in pertinent literary works (*16*). The purity of CS and FS was analyzed by HPLC and was estimated to be greater than 96.0 and 97.5%, respectively.

LDL Oxidation Assay. LDL was isolated from healthy male volunteers in accordance with the procedure of Chung et al. (*17*). In-vitro-induced oxidation of the isolated LDL was performed in accordance with the procedure of Hirano et al. (*18*) with a slight modification. In brief, LDL (final concentration of 100 μg/mL) was incubated for 5 h at 37 °C with freshly prepared V70 (600 μmol/L) or $CuSO_4$ (10 μmol/L) in a spectrophotometer (DU640; Beckman Coulter, Fullerton, CA) that was equipped with a 12-position automatic cell changer. LDL oxidation was monitored by recording the change at 234 nm (formation of conjugated dienes) at 5 min intervals. LDL protein was determined using the BCN protein assay kit (Pierce, Rockford, IL). The time required to yield 50% of the maximum conjugated diene formation was plotted as a function of the standard antioxidant concentration and used as a standard curve to determine the antioxidative capacity of a test sample relative to the standard antioxidant. Gallic acid and Trolox were used as the standard for V70- and copper-induced LDL oxidation, respectively.

Animals and Diets. A total of 48 male apoE-deficient mice (B6.129P2-Apoe^{tm1Unc}/J; Jackson Laboratories, Bar Harbor, ME) aged 6−7 week were divided into 5 groups with 9−10 mice in each group. These mice were fed one of the following diets: (1) control diet, "control" group (n = 9); (2) control diet supplemented with 0.1% (wt/wt) CS and 0.1% FS, "CS + FS (0.2)" group (n = 10); (3) control diet supplemented with 0.2% CS and 0.2% FS, "CS + FS (0.4)" group (n = 10); (4) control diet supplemented with 0.4% FS, "FS" group (n = 9); and (5) control diet supplemented with 1.0% SSE, "SSE" group (n = 10). The control diet comprised the semisynthetic AIN-93G formula with slight modifications (e.g., L-cystine and *tert*-butylhydroquinone were omitted, and soybean oil and casein were replaced with corn oil and vitamin-free casein, respectively). The diet and drinking water were provided ad libitum. The mice were maintained on these diets for 15 weeks, during which their body weight and feed consumption were monitored at weekly intervals. All animal procedures were approved by the institutional ethical review board for animal research.

Analysis of Aortic Atherosclerotic Lesions. Quantification of atherosclerosis was performed by calculating the lesion area in the aortic sinus in accordance with the method of Paigen et al. (*19*). In brief, after 15 weeks of dietary intervention, the mice were fasted overnight, and blood was then collected from the inferior vena cava. The heart and entire aorta were perfused with saline; subsequently, the heart along with the ascending aorta was dissected and embedded in the OCT compound. Cross sections of the heart (thickness of 3 μm) passing through the aortic sinus were prepared, and a section where the aortic valves were most prominent was analyzed. The section was stained with Oil red O and counterstained with hematoxylin. Images of each section were directly captured using a CCD camera attached to a binocular stereomicroscope, and the lesioned area (intimal thickening) was manually determined in accordance with the method of Tangirala

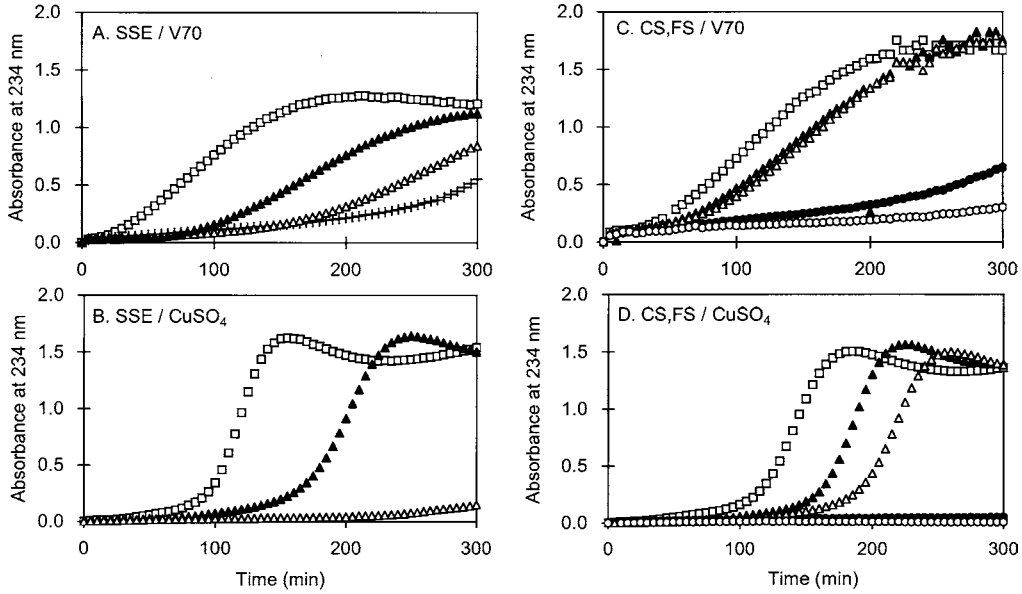

Figure 2. SSE and serotonin derivatives inhibit V70- and $CuSO_4$-induced LDL oxidation (conjugated diene formation) in a dose-dependent manner. Effects of SSE on V70-induced oxidation (A) and $CuSO_4$-induced oxidation (B). (A) Control (□), 0.2 mg/mL SSE (▲), 0.6 mg/mL SSE (△), and 1.2 mg/mL SSE (+). (B) Control (□), 0.3 mg/mL SSE (▲), and 0.5 mg/mL SSE (△). Effects of serotonin derivatives on V70-induced oxidation (C) and $CuSO_4$-induced oxidation (D). (C and D) Control (□), 1 μM CS (▲), 10 μM CS (●), 1 μM FS (△), 10 μM FS (○).

et al. (*20*) by using WinROOF image-analysis software (Mitani-Corp, Tokyo, Japan).

Plasma Lipid and Oxidation Biomarker Measurements. Overnight-fasted blood samples were obtained as described above, mixed with EDTA, and centrifuged to obtain plasma. The mouse non-HDL fraction was obtained from the plasma by the single-spin gradient ultracentrifugation method (*17*), and the lipoprotein composition of the fraction was confirmed by nonlinear-gradient polyacrylamide gel electrophoresis (Multigel-Lipo 2.4/15.2; Daiichi Pure Chemicals, Tokyo, Japan) followed by Sudan black staining. Plasma triglyceride (TG), total cholesterol (TC), and HDL cholesterol (HDL-C) were determined enzymatically using an automated analyzer (Drychem; FujiFilm Medical, Tokyo, Japan). Thiobarbituric acid reactive substances (TBARS) in the non-HDL fraction and plasma were determined in accordance with the procedure of Ohkawa et al. (*21*). The plasma LPO (lipid peroxide) concentration was determined using an LPO assay kit (Determinar LPO; Kyowa Medex, Tokyo, Japan). Plasma autoantibody titers to oxidized LDL (anti-oxLDL autoantibody) were determined using oLAB ELISA kit (Biomedica, Vienna, Austria) with a slight modification. In brief, aliquots (200 μL/well) of diluted (1:500) mouse plasma samples were added to the manufacturer-provided microtiter plate coated with human copper-oxLDL and incubated overnight at 4°C. After washing, diluted (1:20 000) secondary antibody solution (goat anti-mouse IgG peroxidase conjugate; Sigma−Aldrich) was added at an amount of 100 μL/well. After extensive washing, 100 μL of the substrate solution was added to each well, and the reaction was terminated after 15−25 min. Absorbance was measured at 450 nm by using a Wallac ARVO SX 1420 Multilabel Counter (Perkin−Elmer Life Sciences, Boston, MA).

Analysis of Serotonin Derivatives in Mouse Plasma. Nine C57BL/6J mice (Charles River Japan, Inc., Yokohama, Japan) were housed in light-controlled boxes with an inverted 12-h light−dark cycle (07:00−19:00, dark). The mice were fed with the 1.0% SSE diet (described above) for 10 days and sacrificed at 10:00 ($n = 3$), 14:00 ($n = 3$), and 17:00 ($n = 3$) on the last day of the feeding period. Plasma (40 μL) obtained from the inferior vena cava was mixed with or without 10 μL of enzyme solution (100 units of β-glucuronidase and 20 units of sulfatase in 0.5 mol/L sodium acetate buffer at pH 5.0) and then incubated at 37°C for 1 h. Each mixture was extracted with 60 μL of methanol/TCA (100:20), incubated on ice for at least 30 min, and centrifuged (15 000 rpm, 10 min) subsequently. The supernatant was collected and subjected to HPLC equipped with a coulometric detector (CoulArray system; ESA, Inc., Chelmsford, MA). The conditions for HPLC were the same as described above, and the electrode potentials were set between 0 and 700 mV in 100 mV steps.

Statistical Analysis. Statistical analyses were performed by using ANOVA for post-hoc analysis when applicable. Data shown are mean ± SEM.

RESULTS

Inhibition of In-Vitro-Induced LDL Oxidation by Defatted Safflower Seed Extract. Defatted safflower seeds were extracted with 90% ethanol and further fractionated into five fractions by liquid−liquid extraction as described in the Materials and Methods. The antioxidative activity of each fraction was evaluated in a V70-induced LDL oxidation system. The activity was highly correlated with the total polyphenol content in each fraction ($r = 0.941$). The fraction obtained by extraction with ethanol followed by ethyl acetate (designated as SSE) was the most potent in inhibiting LDL oxidation. For a more careful examination of the SSE activity, LDL (100 μg of protein/mL) was subjected to azo-containing radical initiator- or metal-ion-dependent oxidation by incubation with V70 and $CuSO_4$, respectively. The addition of SSE markedly prolonged the lag phase in both the oxidation systems (parts **A** and **B** of **Figure 2**) in a dose-dependent manner. When the antioxidant activity of SSE was compared with that of other antioxidants with respect to the time required to yield 50% of the maximum conjugated diene formation, 1 g of SSE was found to be almost equivalent to 0.2 g of gallic acid or Trolox in the V70- or copper-induced oxidation system, respectively (**Table 1**).

Analysis of Major Phenolic Components in SSE. To identify the antioxidative principals in SSE, the extract was fractionated using reverse-phase HPLC (**Figure 3**). Two major peaks (Rt 31.8 and 32.3 min) were detected at 290 nm. These constituents were identified as CS and FS, respectively, by using ultraviolet spectrum [$\lambda_{max} = 292.4$ nm (CS) and 293.6 and 312.8 nm (FS)] and mass spectrometric analyses [*m*/*z* 323 (CS) and 353 (FS) $[M + H]^+$]. These constituents were further confirmed by coelution with synthetic materials. Two other constituents that were eluted at 25.6 and 26.4 min were observed to possess UV absorption patterns similar to those of CS and FS. Because

Table 1. Contents of Serotonin Derivatives in SSE, Their Antioxidative Activity against V70- and Copper-Induced LDL Oxidation, and Their Contribution to SSE Activity

		V70-induced LDL oxidation		Cu^{2+}-induced LDL oxidation	
	content in SSE (wt %)	specific activity[a]	contribution to SSE (%)	specific activity[b]	contribution to SSE (%)
SSE[c]		0.2		0.2	
CS[c]	8.8	0.9	37.4	1.0	44.0
FS[c]	7.1	1.0	34.4	1.4	49.7
CS–gluc[c]	6.7	0.05	1.5	NT[c]	NT[c]
FS–gluc[c]	1.5	0.05	0.3	NT[c]	NT[c]

[a] Expressed as gallic acid equivalent (g of GAE/g). [b] Expressed as Trolox equivalent (g of TE/g). [c] SSE, ethanol–ethyl acetate extract of defatted safflower seeds; CS, *N*-(*p*-coumaroyl)serotonin; FS, *N*-feruloylserotonin; CS–gluc, CS–monoglucoside; FS–gluc, FS–monoglucoside; NT, not tested.

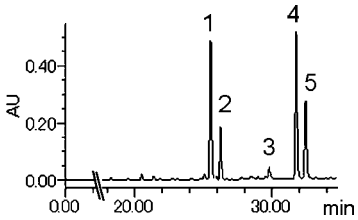

Figure 3. HPLC–UV chromatogram of the ethyl acetate fraction of the ethanolic extract of defatted safflower seeds (SSE). Detection = 290 nm. Peaks: 1, CS–monoglucoside; 2, FS–monoglucoside; 3, 2-hydroxyarctiin; 4, CS; 5, FS.

each constituent yielded a peak with the same retention time as that of CS or FS when hydrolyzed with β-glucosidase (and not with α-glucosidase), the two constituents were considered as monoglucosides of the serotonin derivatives. This conclusion was further supported by the mass spectrometric (CS−monoglucoside, *m*/*z* 485 $[M + H]^+$, 507 $[M + Na]^+$, 323 $[M - C_6H_{10}O_5 + H]^+$; FS−monoglucoside, 515 $[M + H]^+$, 537 $[M + Na]^+$, 353 $[M - C_6H_{10}O_5 + H]^+$) and ^1H NMR (DMSO-d_6, 300 MHz, CS−monoglucoside, anomeric proton at δ 4.73 (1H, d, $J = 7.9$ Hz) and four methines and one methylene between δ 3.1−3.8; FS−monoglucoside, anomeric proton at δ 4.73 (1H, d, $J = 6.9$ Hz) and four methines and one methylene between δ 3.1−3.8) analyses of isolated compounds. An additional constituent that was eluted at 29.8 min was identified as 2-hydroxyarctiin (lignan) by ultraviolet absorption spectrum ($\lambda_{max} = 238.0$ and 278.1 nm) and mass spectrometric analyses (*m*/*z* 551 $[M + H]^+$). The contents of CS, FS, CS−monoglucoside, FS−monoglucoside, and 2-hydroxyarctiin in the extract were 8.8, 7.1, 6.7, 1.5, and 6.1% (wt/wt of SSE), respectively.

In Vitro and ex Vivo Antioxidant Properties of Serotonin Derivatives. Because the serotonin derivatives were considered to be the major phenolic components of SSE, their antioxidative properties were examined in vitro with synthetic compounds. Both CS and FS inhibited V70- or $CuSO_4$-induced LDL oxidation in a dose-dependent manner (parts **C** and **D** of **Figure 2**). The specific activities of both substances were approximately 5 times higher than that of SSE in both oxidation systems. The glucosides of both of the serotonin derivatives were isolated by HPLC and subjected to V70-induced oxidation. Their specific activities were approximately 1/20th of that of their aglycones. The total contribution of the serotonin derivative aglycones to the antioxidative activity of SSE calculated on the basis of their specific activities and their contents was 71.8% in V70-induced oxidation and 93.7% in copper-induced oxidation systems (**Table 1**).

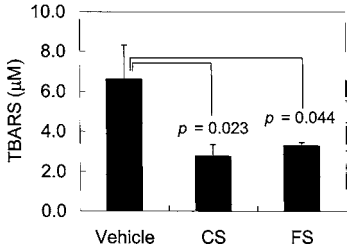

Figure 4. Effect of orally administered serotonin derivatives (100 mg/kg) on the susceptibility of plasma oxidation ex vivo. Plasma oxidation was induced by 100 μM $CuSO_4$ and was measured as the TBARS formation after 120 min of incubation. Bars are mean ± SEM. *p* value; ANOVA, Fisher LSD.

Table 2. Effects of Long-Term (15 Week) Dietary Supplementation of Serotonin Derivatives and SSE on the Plasma Lipid Status in ApoE-Deficient Mice

	plasma lipid (mmol/L)[a]		
groups	TG[b]	TC[b]	HDL-C[b]
control	1.21 ± 0.07	18.2 ± 1.3	0.59 ± 0.06
CS + FS (0.2)	1.14 ± 0.11	15.0 ± 1.4	0.54 ± 0.06
CS + FS (0.4)	1.09 ± 0.09	11.9 ± 0.9[c]	0.74 ± 0.07
FS	0.88 ± 0.08	12.6 ± 1.1[d]	0.43 ± 0.06
SSE	1.13 ± 0.09	12.5 ± 1.1[c]	0.53 ± 0.11

[a] Values are mean ± SEM. [b] TG, triglyceride; TC, total cholesterol; HDL-C, HDL cholesterol. [c] $p < 0.01$ versus "control" (ANOVA, Dunnett). [d] $p < 0.05$ versus "control" (ANOVA, Dunnett).

CS, FS (100 mg/kg each), and vehicle [50% poly(ethylene glycol) 400] were orally administered to C57BL/6 mice ($n = 5$, each). Plasma collected 30 min after ingestion was 10-fold diluted with saline and then subjected to $CuSO_4$ (100 μM) induced oxidation for 120 min at 37 °C. As shown in **Figure 4**, TBARS formation in plasma was significantly suppressed in both serotonin-derivative-administered mice.

Effects of SSE and Serotonin Derivatives on Aortic Lesion Formation in ApoE-Deficient Mice. Because SSE and serotonin derivatives strongly inhibited in-vitro-induced LDL oxidation, we investigated their antiatherosclerotic activity in apoE-deficient mice. At the end of the intervention, no significant difference was observed in the body weights of mice in different groups [29.6 ± 0.6 g in "control", 28.0 ± 0.9 g in "CS + FS (0.2)", 28.2 ± 0.4 g in "CS + FS (0.4)", 27.9 ± 0.6 g in "FS", and 28.1 ± 0.8 g in "SSE"]. Similarly, no significant differences were observed in plasma triglyceride and HDL cholesterol levels. However, the total cholesterol was significantly lower in animals treated with SSE or serotonin derivatives ("safflower-treated mice") (**Table 2**). The extent of atherosclerosis in the aortic sinus of the control and safflower-treated mice was evaluated. The mean lesioned area was significantly smaller in all of the safflower-treated mice; 29.2% ($p < 0.05$), 53.3% ($p < 0.01$), 43.5% ($p < 0.01$), and 79.7% ($p < 0.01$) reduction was observed in mice of the "CS + FS (0.2)", "CS + FS (0.4)", "FS", and "SSE", respectively (**Figure 5A**). Although the total content of the serotonin derivatives in the "SSE" diet (0.21%; aglycone basis) was similar to that of the "CS + FS (0.2)" diet, SSE-treated mice showed significantly less extensive lesions than the mice fed with the diet containing pure serotonin derivatives.

Effects of SSE and Serotonin Derivatives on Plasma Lipid Peroxidation and Anti-oxLDL Autoantibody Titers. To study the antiatherosclerotic mechanism of SSE or serotonin derivatives, some oxidative biomarkers were examined in the saf-

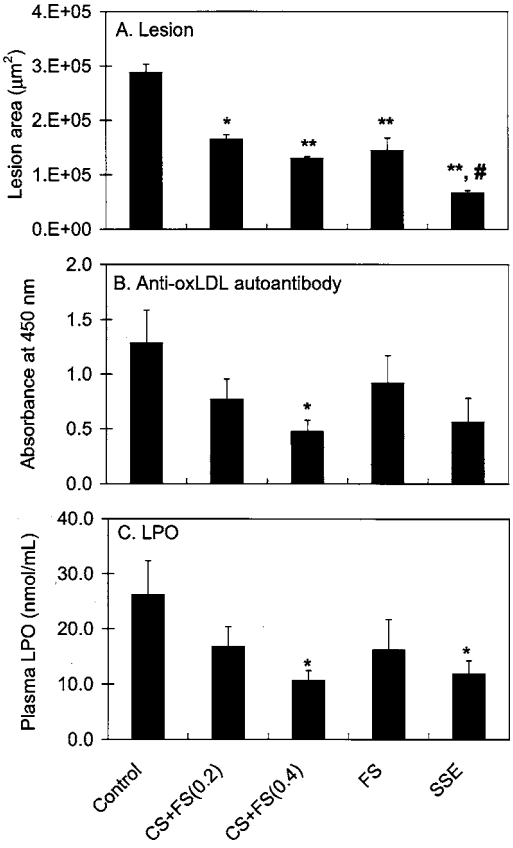

Figure 5. Effects of the long-term (15 week) dietary supplementation of SSE and serotonin derivatives on atherosclerotic lesion formation (A), plasma anti-oxLDL autoantibody (B), and plasma lipid peroxides (LPO) (C) in apoE-deficient mice. Bars are mean ± SEM. (A) (*) $p < 0.05$, (**) $p < 0.01$ versus "control", and (#) $p < 0.05$ versus "CS + FS (0.2)" (ANOVA, Tukey–Kramer). (B and C) (*) $p < 0.05$ versus "control" (ANOVA, Dunnett).

flower-treated apoE-deficient mice. When the non-HDL fractions (VLDL−LDL) separated from the safflower-treated mice were subjected to V70-induced oxidation, no prolonged or even slightly shorter lag time was observed [33.0 ± 2.6 min in "control", 28.9 ± 1.4 in "CS + FS (0.2)", 25.6 ± 1.2 in "CS + FS (0.4)" ($p < 0.05$ versus "control"), 26.2 ± 1.3 in "FS", and 27.6 ± 2.1 in "SSE"]. However, the mean TBARS values in the lipoprotein fraction showed a tendency to decrease in all of the safflower-treated groups except in the "CS + FS (0.2)" diet; however, this decrease was not significant because of considerable variations [0.21 ± 0.11 nmol/mg protein in "control", 0.23 ± 0.08 in "CS + FS (0.2)", 0.11 ± 0.07 in "CS + FS (0.4)", 0.18 ± 0.11 in "FS", and 0.13 ± 0.05 in "SSE"]. There was a significant reduction ($p < 0.05$ versus "control") in the plasma anti-oxLDL autoantibody titers in the "CS + FS (0.4)" diet (**Figure 5B**). Although not significant, relatively lower autoantibody titers were also observed in the plasma of mice in the rest of safflower-treated groups. The plasma level of LPO showed the same tendency; a significant reduction ($p < 0.05$ versus "control") was observed in the "CS + FS (0.4)" and "SSE" diets (**Figure 5C**).

Biological Availability of Serotonin Derivatives. We questioned whether the antiatherogenic and antioxidative effects observed by the oral intake of SSE or serotonin derivatives could be attributed to the absorption of serotonin derivatives into the circulation. At 3, 7, and 10 h after the onset of the dark period, blood was collected from the C57BL/6J mice fed a diet containing 1% SSE ad libitum. Plasma was separated from the blood and treated with or without β-glucuronidase and sulfatase and then analyzed for serotonin derivatives and their metabolites by using the HPLC−ECD system. Both serotonin derivatives were observed to be present in the plasma in both intact and conjugated forms (**Table 3**). The total concentration of the intact aglycones (CS + FS) was maintained above 1 μmol/L at least from 3 to 7 h; however, a majority of the serotonin derivatives were detected as conjugated metabolites (glucuronides and/or sulfates). Both aglycones accounted for approximately 10% of the total detectable plasma serotonin derivatives and further decreased to 5% toward the end of the feeding period.

DISCUSSION

The present study demonstrated that the ethanol−ethyl acetate extract of defatted safflower seeds (SSE) inhibited both V70- and Cu^{2+}-induced LDL oxidation. Serotonin derivatives such as CS, FS, and their glucosides were identified as the major phenolics in the extract. Chemically synthesized serotonin derivatives strongly inhibited in-vitro-induced LDL oxidation and decreased plasma susceptibility to oxidation ex vivo. SSE and serotonin derivatives attenuated the atherosclerosis development in apoE-deficient mice and concomitantly reduced the total cholesterol, LPO, and anti-oxLDL autoantibody titers in their plasma. These serotonin derivatives were detectable in both intact and conjugated forms in the plasma of C57BL/6J mice fed with a diet containing SSE. To the best of our knowledge, this is the first paper that demonstrates the pharmacological action as well as the active compounds of safflower seed extract in spontaneous disease model animals.

Both SSE and serotonin derivatives inhibited LDL oxidation induced by either V70 (a free-radical-generating system) or $CuSO_4$ (a metal-ion-dependent lipid peroxidation system) (**Figure 2**). In either oxidation system, the antioxidative activity of the aglycones of the serotonin derivatives accounted for >70% of that of SSE (**Table 1**). The glucosides exhibited considerably lower specific activity (1/20th) than the aglycones. These results indicate that the majority of the antioxidative activity against in-vitro-induced LDL oxidation in SSE is derived from aglycones of the serotonin derivatives. Consistent with our findings, Zhang et al. (*22*) reported the absence of significant DPPH-scavenging activity in both glucosides in

Table 3. Appearance of Serotonin Derivatives in the Plasma of C57BL/6J Mice When Fed on a Diet Containing 1% SSE[a]

	CS (μmol/L)[b]			FS (μmol/L)[b]		
	3 h	7 h	10 h	3 h	7 h	10 h
intact (A)[c]	0.60 ± 0.13	0.81 ± 0.23	0.25 ± 0.08	0.56 ± 0.09	0.46 ± 0.08	0.19 ± 0.06
conjugates[d]	6.57 ± 1.36	7.53 ± 1.30	5.35 ± 1.56	4.91 ± 0.94	4.60 ± 0.66	3.77 ± 0.86
total (B)[e]	7.17 ± 1.42	8.33 ± 1.10	5.60 ± 1.62	5.47 ± 1.01	5.05 ± 0.72	3.96 ± 0.91
A/B (%)	8.4	9.7	4.5	10.3	9.0	4.7

[a] Nine C57BL/6J mice were fed with 1% (wt/wt) SSE diet ad libitum; the mice were sacrificed at 3 h ($n = 3$), 7 h ($n = 3$), and 10 h ($n = 3$) after the onset of the dark period; and plasma was obtained. The plasma was treated with or without deconjugating enzymes prior to HPLC analysis. [b] Values are mean ± SEM. [c] Without enzymatic digestion. [d] Total (B) minus intact (A) (glucuronides and/or sulfates). [e] Digested with β-glucuronidase and sulfatase.

which the 5-OH groups of the indole ring were glycosylated. Further, the result is suggestive of the major contribution of the serotonin moiety with the free 5-OH group with respect to the antioxidative activity of the serotonin derivatives.

Because the synthetic serotonin derivatives themselves significantly attenuated lesion formation in apoE-deficient mice, serotonin derivatives were considered, at least in part, if not all, antiatherosclerotic principals in SSE. When almost equal concentrations of SSE and pure serotonin derivatives (CS + FS) were administered to the mice on a total serotonin derivative aglycone basis (0.2%), the effect of reducing the lesion in the synthetic serotonin derivative groups was significantly lower than that in the SSE group (**Figure 5A**). This may suggest that SSE contains other antiatherogenic compounds besides the serotonin derivatives. The serotonin derivatives reduced the lesion area in a dose-dependent manner. Administration of 0.4% of the serotonin derivatives resulted in almost comparable lesion reduction irrespective of whether the serotonin derivatives were administered as a mixture (CS + FS) or alone (FS). CS and FS may be equipotent in antiatherogenicity as well as in their in vitro antioxidative activity described above.

The lesion-reducing effects were associated with parallel reductions in plasma total cholesterol (**Table 2**), LPO (**Figure 5C**), and anti-oxLDL autoantibody titers (**Figure 5B**). In conjunction with the result of this study, the reported hypolipidemic effects of safflower seed extracts or their phenolic constituents on cholesterol-fed rats (*8*, *23*) may enable the deduction of some beneficial effects of safflower seeds on lipid metabolism and atherogenesis; however, in three other experiments that we performed, neither SSE nor the serotonin derivatives reproduced the hypocholesterolemic effect, while reduction in the atherosclerotic lesion area was similarly observed (data not shown). Thus, the hypocholesterolemic effect observed in this study may not be essential for the expression of antiatherosclerotic action. The association of hypocholesterolemic activity in safflower seeds with its antiatherosclerotic activity seems to require a more careful assessment.

A number of studies have suggested that higher titers of autoantibodies to oxidative-modified LDL are found in patients with cardiovascular diseases and also in apoE-deficient mice (*24*). Circulating autoantibody titers have been shown to correlate with oxidized LDL content in atherosclerotic lesions of LDL-receptor-deficient mice (*25*). Our data on plasma lipid peroxide and anti-oxLDL autoantibody titers in conjunction with the ex vivo antioxidative activity of CS and FS provide an indication of the suppressive effect of the serotonin derivatives on the formation of oxidized LDL through their in vivo antioxidative action. However, we failed to demonstrate the prolongation of the lag time in the non-HDL fraction obtained from the safflower-treated apoE-deficient mice when subjected to V70-induced oxidation. This may suggest that serotonin derivatives are not incorporated into lipoprotein particles and removed from the lipoprotein fraction by the fractionation procedures or metabolized and cleared from plasma after overnight fasting.

Stocker and O'Halloran (*26*) and Waddington et al. (*27*) reported the lack of an effect of red wine polyphenols on lipid oxidation, while development of atherosclerosis in apoE-deficient mice was partially inhibited. They suggested that arterial lipoprotein oxidation could be dissociated from atherogenesis, and activities other than inhibition of lipid peroxidation might be more important. Antioxidative activity against lipoprotein oxidation of serotonin derivatives is the only mechanism examined here, and we do consider it relevant to the protective effect of SSE; however, we do not exclude the possibility of involvement of the activities of serotonin derivatives other than antilipoprotein oxidation because effects on inflammatory cytokine production (*12*) or nitric oxide generation (*11*) have been suggested. Future studies are needed to unveil other potential beneficial activities in serotonin derivatives and to elucidate the in vivo relevance of them.

We demonstrated for the first time the biological availability of the serotonin derivatives by their detection in the plasma of SSE-fed mice (**Table 3**). The plasma level of total serotonin derivative aglycones (CS + FS) reached over 1 μmol/L, in which the concentration was shown to inhibit LDL oxidation in vitro (parts **C** and **D** of **Figure 2**). Thus, it seems presumable that the serotonin derivatives might have prevented LDL oxidation in vivo by scavenging the ROS present in the aqueous milieu of plasma and/or interstitial fluid of the arterial wall.

The majority of the absorbed serotonin derivatives was likely to be metabolized to conjugated forms. The biological properties of some flavonoid conjugates have been shown to depend upon the position of the substitution (*28*). At present, the position of the substitution in the serotonin derivatives has not been determined. The biological activity of these metabolites remained to be evaluated.

In conclusion, the ethanol−ethyl acetate extract of defatted safflower seeds is preventive against atherosclerotic development, and its major and novel subclass of polyphenols, serotonin derivatives, are responsible for its antioxidative and, at least in part, antiatherosclerotic activities. SSE or the serotonin derivatives may be beneficial in preventing human cardiovascular diseases.

ACKNOWLEDGMENT

The authors are grateful to Narihide Matsuzaki of J-Oil Mills, Inc. (Tokyo, Japan) for supplying defatted safflower seeds and to Yoshiko Yamazaki for providing help with HPLC analysis. The technical assistance provided by Tomoka Yamano with respect to the biochemical measurements and animal experiments is greatly appreciated.

LITERATURE CITED

(1) Miura, Y.; Chiba, T.; Tomita, I.; Koizumi, H.; Miura, S.; Umegaki, K.; Hara, Y.; Ikeda, M.; Tomita, T. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *J. Nutr.* **2001**, *131*, 27−32.

(2) Yamakoshi, J.; Kataoka, S.; Koga, T.; Ariga, T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **1999**, *142*, 139−149.

(3) Kamada, C.; da Silva, E. L.; Ohnishi-Kameyama, M.; Moon, J. H.; Terao, J. Attenuation of lipid peroxidation and hyperlipidemia by quercetin glucoside in the aorta of high cholesterol-fed rabbit. *Free Radical Res.* **2005**, *39*, 185−194.

(4) Enkhmaa, B.; Shiwaku, K.; Katsube, T.; Kitajima, K.; Anuurad, E.; Yamasaki, M.; Yamane, Y. Mulberry (*Morus alba* L.) leaves and their major flavonol quercetin 3-(6-malonylglucoside) attenuate atherosclerotic lesion development in LDL receptor-deficient mice. *J. Nutr.* **2005**, *135*, 729−734.

(5) Kurosawa, T.; Itoh, F.; Nozaki, A.; Nakano, Y.; Katsuda, S.; Osakabe, N.; Tsubone, H.; Kondo, K.; Itakura, H. Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. *Atherosclerosis* **2005**, *179*, 237−246.

(6) Fuhrman, B.; Buch, S.; Vaya, J.; Belinky, P. A.; Coleman, R.; Hayek, T.; Aviram, M. Licorice extract and its major polyphenol glabridin protect low-density lipoprotein against lipid peroxidation: in vitro and ex vivo studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am. J. Clin. Nutr.* **1997**, *66*, 267−275.

(7) Sakamura, S.; Terayama, Y.; Kawakatsu, S.; Ichihara, A.; Saito, H. Conjugated serotonins related to cathartic activity in safflower seeds (*Carthamus tinctorius* L.). *Agric. Biol. Chem.* **1978**, *42*, 1805−1806.

(8) Cho, S. H.; Lee, H. R.; Kim, T. H.; Choi, S. W.; Lee, W. J.; Choi, Y. Effects of defatted safflower seed extract and phenolic compounds in diet on plasma and liver lipid in ovariectomized rats fed high-cholesterol diets. *J. Nutr. Sci. Vitaminol.* **2004**, *50*, 32−37.

(9) Bae, S.; Shim, S.; Park, Y.; Lee, J.; Chang, E.; Choi, S. Cytotoxicity of phenolic compounds isolated from seeds of safflower (*Carthamus tinctorius* L.) on cancer cell lines. *Food Sci. Biotechnol.* **2002**, *11*, 140−146.

(10) Takii, T.; Hayashi, M.; Hiroma, H.; Chiba, T.; Kawashima, S.; Zhang, H. L.; Nagatsu, A.; Sakakibara, J.; Onozaki, K. Serotonin derivative, *N*-(*p*-coumaroyl)serotonin, isolated from safflower (*Carthamus tinctorius* L.) oil cake augments the proliferation of normal human and mouse fibroblasts in synergy with basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF). *J. Biochem.* **1999**, *125*, 910−915.

(11) Hotta, Y.; Nagatsu, A.; Liu, W.; Muto, T.; Narumiya, C.; Lu, X.; Yajima, M.; Ishikawa, N.; Miyazeki, K.; Kawai, N.; Mizukami, H.; Sakakibara, J. Protective effects of antioxidative serotonin derivatives isolated from safflower against postischemic myocardial dysfunction. *Mol. Cell. Biochem.* **2002**, *238*, 151−162.

(12) Kawashima, S.; Hayashi, M.; Takii, T.; Kimura, H.; Zhang, H. L.; Nagatsu, A.; Sakakibara, J.; Murata, K.; Oomoto, Y.; Onozaki, K. Serotonin derivative, *N*-(*p*-coumaroyl)serotonin, inhibits the production of TNF-α, IL-1α, IL-1*β*, and IL-6 by endotoxin-stimulated human blood monocytes. *J. Interferon Cytokine Res.* **1998**, *18*, 423−428.

(13) Nagatsu, A.; Zhang, H.; Mizukami, H.; Okuyama, H.; Sakakibara, J.; Tokuda, H.; Nishino, H. Tyrosinase inhibitory and anti-tumor promoting activities of compounds isolated from safflower (*Carthamus tinctorius* L.) and cotton (*Gossypium hirsutum* L.) oil cakes. *Nat. Prod. Lett.* **2000**, *14*, 153−158.

(14) Singleton, V.; Orthofer, R.; Lamuela-Raventós, R. M. Analysis of total polyphenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.

(15) Lyon, C. K.; Gumbmann, M. R.; Betschart, A. A.; Robbins, D. J.; Saunders, R. M. Removal of deleterious glucosides from safflower meal. *J. Am. Oil Chem. Soc.* **1979**, *56*, 560−564.

(16) Sakamura, S.; Terayama, Y.; Kawakatsu, S.; Ichihara, A.; Saito, H. Conjugated serotonins and phenolic constituents in safflower seed (*Carthamus tinctorius* L.). *Agric. Biol. Chem.* **1980**, *44*, 2951−2954.

(17) Chung, B. H.; Segrest, J. P.; Ray, M. J.; Brunzell, J. D.; Hokanson, J. E.; Krauss, R. M.; Beaudrie, K.; Cone, J. T. Single vertical spin density gradient ultracentrifugation. *Methods Enzymol.* **1986**, *128*, 181−209.

(18) Hirano, R.; Kondo, K.; Iwamoto, T.; Igarashi, O.; Itakura, H. Effects of antioxidants on the oxidative susceptibility of low-density lipoprotein. *J. Nutr. Sci. Vitaminol.* **1997**, *43*, 435−444.

(19) Paigen, B.; Morrow, A.; Holmes, P. A.; Mitchell, D.; Williams, R. A. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **1987**, *68*, 231−240.

(20) Tangirala, R. K.; Rubin, E. M.; Palinski, W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **1995**, *36*, 2320−2328.

(21) Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351−358.

(22) Zhang, H. L.; Nagatsu, A.; Watanabe, T.; Sakakibara, J.; Okuyama, H. Antioxidative compounds isolated from safflower (*Carthamus tinctorius* L.) oil cake. *Chem. Pharm. Bull.* **1997**, *45*, 1910−1914.

(23) Moon, K.; Back, S.; Kim, J.; Jeon, S.; Lee, M.; Choi, M. Safflower seed extract lowers plasma and hepatic lipids in rats fed high-cholesterol diet. *Nutr. Res.* **2001**, *21*, 895−904.

(24) Palinski, W.; Horkko, S.; Miller, E.; Steinbrecher, U. P.; Powell, H. C.; Curtiss, L. K.; Witztum, J. L. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low-density lipoprotein in human plasma. *J. Clin. Invest.* **1996**, *98*, 800−814.

(25) Tsimikas, S.; Palinski, W.; Witztum, J. L. Circulating autoantibodies to oxidized LDL correlate with arterial accumulation and depletion of oxidized LDL in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **2001**, *21*, 95−100.

(26) Stocker, R.; O'Halloran, R. A. Dealcoholized red wine decreases atherosclerosis in apolipoprotein E gene-deficient mice independently of inhibition of lipid peroxidation in the artery wall. *Am. J. Clin. Nutr.* **2004**, *79*, 123−130.

(27) Waddington, E.; Puddey, I. B.; Croft, K. D. Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. *Am. J. Clin. Nutr.* **2004**, *79*, 54−61.

(28) Williamson, G.; Barron, D.; Shimoi, K.; Terao, J. In vitro biological properties of flavonoid conjugates found in vivo. *Free Radical Res.* **2005**, *39*, 457−469.

Received for review January 27, 2006. Revised manuscript received May 8, 2006. Accepted May 10, 2006.

JF060254P