JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidant Activity of Isoflavones and Their Major Metabolites Using Different in Vitro Assays

CORINNA E. RÜFER† AND SABINE E. KULLING*,‡

Institute of Nutritional Physiology, Federal Research Centre for Nutrition and Food, Haid-und-Neu-Strasse 9, 76131 Karlsruhe, and Institute of Nutritional Science, Department of Food Chemistry, University of Potsdam, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany

Isoflavone phytoestrogens found mainly in soybeans and clover are widely studied phytochemicals. Genistein and daidzein, the major isoflavones found in soy, have received the most attention. However, they undergo extensive metabolism in the intestine and the liver, which might affect their biological properties, e.g. their antioxidant capacities. Furthermore, the biological activities of other naturally occurring isoflavones, for instance, glycitein from soy or biochanin A from red clover, have not yet been studied in detail. The aim of this study was to investigate the antioxidant activities of six naturally occurring isoflavones and their corresponding oxidative and bacterial metabolites. The oxygen radical absorbance capacity assay as well as the in vitro oxidation of low density lipoproteins with the conjugated diene and the thiobarbituric acid reacting substances formation as end points were used. The oxidative metabolites of genistein and daidzein as well as equol exhibited the highest antioxidant activities in all three assays. With few exceptions, they were more effective than the positive controls quercetin and ascorbic acid. Formononetin, the 4′-*O*-methyl ether of daidzein, showed the lowest antioxidant property. Because the antioxidant efficacy of isoflavones as effective antioxidants is evident at concentrations well within the range found in the plasma of subjects consuming soy products, this biological activity could be of physiological relevance.

KEYWORDS: Isoflavones; bacterial and oxidative metabolites; antioxidant activity; ORAC assay; in vitro oxidation of low-density lipoproteins

INTRODUCTION

Phytoestrogens of the isoflavone family are found in numerous plants used for human and animal nutrition. They are most abundant in soybeans but are also present in appreciable amounts in a variety of beans, sprouts, and legumes. Animal feed such as clover or alfalfa is a rich source of isoflavones as well. In **Figure 1**, the structures of the most common isoflavones are depicted. Their occurrence varies between different plants; for example, soybeans contain mostly daidzein (DAI), genistein (GEN), and glycitein (GLY) whereas red clover is high in formononetin (FORM), biochanin A (BCA), and prunetin (PRU) (*1*, *2*).

Isoflavone intake is associated with a broad variety of properties beneficial to human health. GEN and DAI have received considerably more attention than other naturally occurring isoflavones, e.g. GLY, BCA, FORM, and PRU. Furthermore, the metabolites formed are the key to understanding the beneficial effects of isoflavones since it is the metabolite, rather than the parent compound, to which cells are predominantly exposed. The colonic microflora play another important role in the metabolism of isoflavones. It has been reported that DAI is converted by the gut microflora to dihydrodaidzein (DHD), which can be further metabolized to both equol and *O*-desmethylangolensin (O-DMA) (see **Figure 1**). GEN is first reduced by gut bacteria to dihydrogenistein (DHG), followed by a cleavage of the C-ring to form 6′-hydroxy-*O*-desmethylangolensin (6′-OH-ODMA) (see **Figure 1**), which can be further degraded to 4-hydroxyphenyl-2-propionic acid. Decarboxylation can then lead to the putative metabolic end product 4-ethylphenol (*3*−*5*). For the phase I metabolism, Kulling et al. have been able to show that DAI and GEN undergo biotransformation catalyzed by cytochrome P450 enzymes in vitro and in vivo (*6*, *7*). Several mono- and dihydroxylated DAI and GEN metabolites—predominantly in the C-6, -8, and -3′ positions—have been identified in human urine (see **Figure 1**) (*5*, *7*).

All described metabolites have been identified in human plasma and urine (*8*). However, data about their biological properties are scarce. It is generally believed that many of the beneficial effects of isoflavones might be related to their antioxidant activity, but little attention has been paid to the antioxidant capacity of other naturally occurring isoflavones also found in plasma and urine, e.g. GLY, as well as the formed metabolites. Therefore, the aim of this study was to analyze

* To whom correspondence should be addressed. Tel: +49-33200/88-580. Fax: +49-33200/88-582. E-mail: kulling@rz.uni-potsdam.de.
† Federal Research Centre for Nutrition and Food.
‡ University of Potsdam.

10.1021/jf053112o CCC: $33.50
Published on Web 03/18/2006

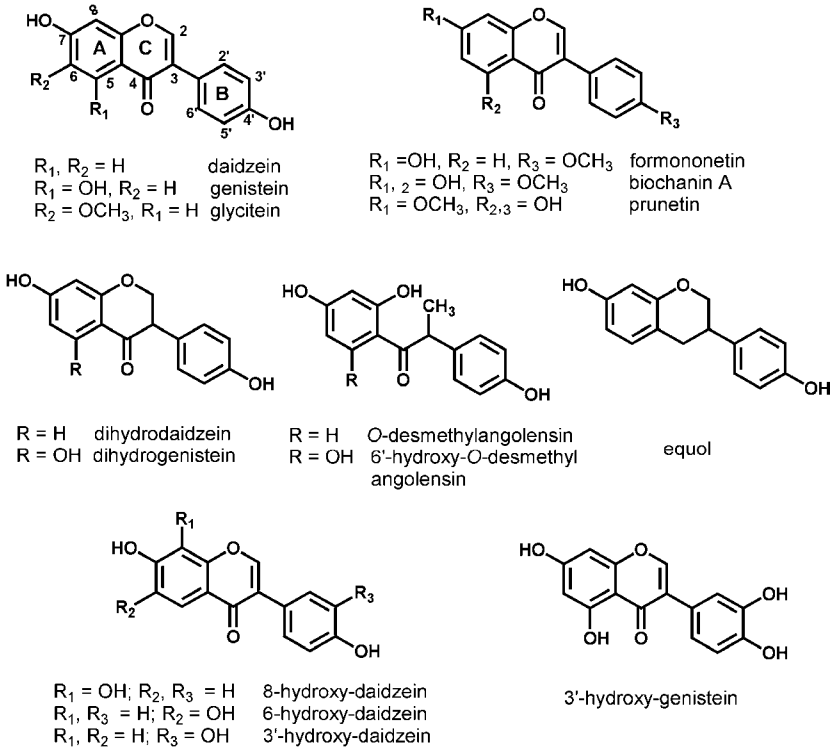

Figure 1. Chemical structures of isoflavones and their oxidative and bacterial metabolites.

the antioxidant properties of naturally occurring isoflavones as compared to their corresponding metabolites. The goal was to establish structural features, which enhance the antioxidant activity. Furthermore, the underlying mechanisms of their antioxidant properties were to be determined by using different assays—the oxygen radical absorbance capacity (ORAC) assay as well as the in vitro oxidation of low-density lipoproteins (LDL) with the conjugated diene and the thiobarbituric acid reacting substances (TBARS) formation as end points. Quercetin (QUER), a dietary flavonoid, and ascorbic acid (ASC)—both known effective antioxidants—were used as positive controls.

MATERIALS AND METHODS

Materials. GEN and DAI were purchased from TCI Tokyo Casei (Tokyo, Japan), and GLY was purchased from LC Laboratories (Woburn, MA). Their corresponding oxidative and bacterial metabolites as well as PRU, FORM, and BCA were obtained from Plantech (Reading, United Kingdom). Trolox was purchased from Sigma-Aldrich (Taufkirchen, Germany), R-phycoerythrin (PE) was from Molecular Probes Europe BV (Leiden, Netherlands), and ABAP [2,2′-azobis(2-amidinopropane)] was from Polysciences Inc. (Warrington, PE). 1,1,3,3-Tetraethoxypropane was obtained from Fluka (Taufkirchen, Germany), the CHOD-PAP enzymatic test kit was from Roche Diagnostics GmbH (Mannheim, Germany), and the Chelex 100 ion-exchange resin was from Bio-Rad (Munich, Germany). All other chemicals used were obtained from Fluka.

ORAC Assay. The antioxidant activity was determined following a procedure similar to that described by Cao and Prior (*8*). The reaction was carried out in 75 mM phosphate buffer (pH 7.0), and the final reaction mixture was 300 μL. Concentrations (2, 4, 6, and 8 μM) of Trolox (used as standards), 15 μL of antioxidant working solution (in buffer) (see below), or buffer alone (blank) as well as 250 μL of 15.5 nM PE were mixed in 96 well plates (Greiner, Frickenhausen, Germany) and maintained at 37 °C for 5 min. The oxidation reaction was started by the addition of 35 μL of 275 mM ABAP to each well. The fluorescence of PE was excited at $\lambda = 495$ nm, and the fluorescence emission was detected at $\lambda = 575$ nm. The decay of PE fluorescence was monitored every 3 min at 37 °C until the fluorescence of the last reading had declined to less than 5% of the first reading (for up to 2.5 h) using a microplate reader (Tecan Spectra Fluor Plus, Crailsheim, Germany). The microplate was shaken prior to each reading. Test compounds were dissolved in a little volume of dimethyl sulfoxide (about 1/10 of the final volume), and ethanol was added until a final concentration of 10 mM (stock solution) was reached. Working solutions were prepared by diluting daily stock solutions with phosphate buffer to at least four different concentrations in the range of the Trolox calibration curve. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. For quantification, the raw data were exported to an Excel (Microsoft) sheet. After normalizing the antioxidant curves (fluorescence versus time) to the curve of the blank, the areas under the fluorescence decay curves (AUC) were calculated. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. ORAC values for the test compounds were expressed as Trolox equivalents by using the calibration curve calculated for each assay. For all compounds, a linear correlation between net AUC and concentration was obtained. One Trolox equivalent equaled the net protection produced by 1 mM Trolox.

Lipoprotein Separation. Venous blood was drawn from overnight-fasting adults into polypropylene tubes containing 10% EDTA solution (pH 7.4) to give a final concentration of 0.1% EDTA. Plasma was immediately separated by centrifugation at 1500*g* for 10 min at 4 °C. A sucrose solution (75%) was added to give a final concentration of 1.5 g sucrose/L plasma. Aliquots were stored at −80 °C in the dark until LDL preparation. LDL ($\delta = 1.019-1.063$ g/mL) was prepared by ultracentrifugation using a single step discontinuous gradient in KBr solution in a Beckmann 50.4 Ti rotor (Beckmann, Munich, Germany) at 35000*g* and 15 °C for 2 h according to Kleinveld et al. (*9*). The LDL solution was stored at 4 °C under N_2 in the dark for up to 3 days. Before oxidation, 0.5−1.0 mL of the LDL stock solution was desalted and made EDTA free by gel filtration using PD 10 columns (Amersham Biosciences Europe GmbH, Freiburg, Germany) with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM NaH_2PO_4, and 10 mM Na_2HPO_4, pH 7.3) as the eluent. To remove contaminating transition metal ions, the PBS solution was pretreated with 5 g/L of Chelex 100 ion exchange resin. The LDL concentration was determined by measuring total cholesterol with the commercial CHOD-PAP enzymatic test kit. LDL concentration is expressed as micromolar, assuming an LDL molecular mass of 2.5 MDa and a cholesterol content of 31.6% (w/w) (*10*).

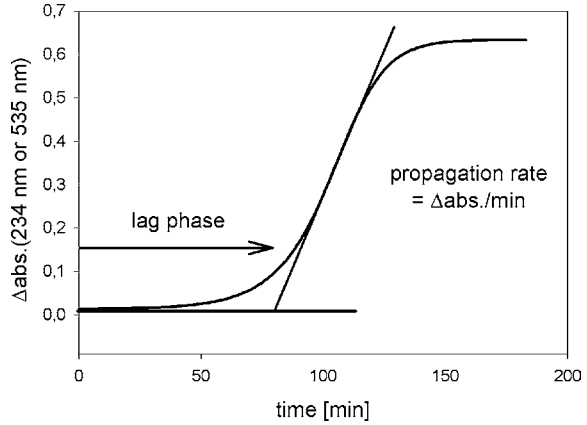

Figure 2. Determination of the oxidation lag phase, which is defined as the interval between the initiation of the oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase.

In Vitro Oxidation of LDL−Conjugated Diene Formation. The LDL oxidation process was followed by determining the formation of conjugated dienes through measuring the absorption at $\lambda = 234$ nm in a mircoplate reader (Tecan Spectra Fluor Plus). The reaction was carried out in PBS, and the final reaction mixture was 250 μL. In each well of a 96 well plate, 235 μL of a 0.1 μM LDL solution was mixed with 5 μL of antioxidant working solution (see below) in ethanol or ethanol alone (blank). The reaction was started by the addition of 10 μL of a 500 μM $CuSO_4$ solution (20 μM final concentration). The kinetics of oxidation were monitored by recording the absorption at $\lambda = 234$ nm every 3 min up to 210 min at 25 °C. Stock solutions of the test compounds were prepared as described for the ORAC assay. Working solutions were achieved by diluting daily stock solutions with ethanol to at least four different concentrations. All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. For quantification, after the antioxidant curves were normalized to the curve of the blank, oxidation kinetics were analyzed on the basis of the oxidation lag phase, which was defined as the interval between the initiation of the oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase (see **Figure 2**). For easier comparison of the antioxidant capacities of the test compounds, we calculated the antioxidant concentration (μM), which is needed to prolong the lag phase by 50% (EC_{50}).

In Vitro Oxidation of LDL−TBARS Formation. Oxidation of LDL was performed in plastic tubes with a final volume of 4 mL. A 3.84 mL amount of a 0.1 μM LDL solution and 4 μL of antioxidants in ethanol or ethanol alone (blank) were mixed. The reaction was started by adding 160 μL of a 500 μM $CuSO_4$ (20 μM final concentration). After mixing, the loosely capped tubes were incubated at 37 °C for different time periods (0, 1, 2, 3, 4, 5, and 6 h) in a shaking water bath. For the absorbance assay of TBARS formation, an aliquot (500 μL) was mixed with 20 μL of 5% butylated hydroxytoluene (in ethanol), 375 μL of 20% trifluoracetic acid, and 500 μL of 1.44% thiobarbituric acid (in 0.5 M KOH). The tubes were heated at 95 °C for 45 min and then cooled on ice. After centrifugation at 14000*g* for 10 min, the supernatant was transferred to a plastic cuvette and 100 μL of 20% sodium dodecyl sulfate was added to reduce turbidity. Absorbance was measured as the difference between $\lambda = 532$ and 600 nm to eliminate light scattering interference. Measurements were carried out in a Perkin-Elmer Lambda 15 UV/vis spectrophotometer (Rodgau, Juegesheim, Germany). TBARS were quantified using a standard curve of MDA, generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane. Stock and working solutions of the test compounds were prepared as described for the conjugated diene formation. At least three independent assays were performed for each sample. Oxidation kinetics and EC_{50} values were analyzed in the same way as described for the formation of conjugated dienes.

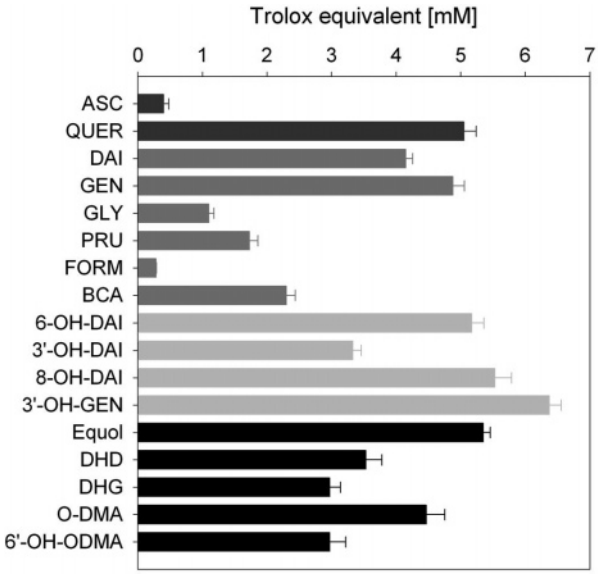

Figure 3. Trolox equivalents (mM) for the tested isoflavones and their corresponding oxidative and bacterial metabolites as compared to QUER and ASC determined with the ORAC assay. Bars show means ± standard deviations of at least triplicate experiments.

RESULTS

The ORAC assay and the in vitro oxidation of LDL were used to determine the antioxidant capacity of isoflavones and their corresponding metabolites. The ORAC assay is based on the detection of chemical damage to PE through the decrease in its fluorescence emission. The loss of PE fluorescence in the presence of radical species, which include peroxyl radicals generated from ABAP, is an index of oxidative damage of the protein. The inhibition of the radical species action by an antioxidant, which is reflected in the protection against the loss of PE fluorescence, is a measure of its antioxidant capacity against radical species. One Trolox equivalent is equivalent to the net protection produced by 1 mM Trolox. The Trolox equivalents for the tested compounds are depicted in **Figure 3**. 3′-Hydroxy-genistein (3′-OH-GEN), 8-hydroxy-daidzein (8-OH-DAI), 6-hydroxy-daidzein (6-OH-DAI), and equol all exhibited higher antioxidant activities than QUER. FORM showed the lowest antioxidant efficacy. All other tested isoflavone compounds were more effective than ASC. The Trolox equivalents for 3′-OH-GEN were 16-fold higher than for ASC.

The effects of the parent isoflavones, of their oxidative and bacterial metabolites, as well as of QUER and ASC on the duration of the lag phase regarding the formation of conjugated dienes in the in vitro oxidation of LDL are depicted in **Figure 4**. The corresponding EC_{50} values are shown in **Figure 5**. For all compounds, a good correlation between antioxidant effect and concentration was obtained. For clarity, only results for 1, 5, and 10 μM are shown. In this assay, again, the oxidative metabolites of GEN and DAI as well as equol are most effective in increasing the lag phase and FORM is least effective. At the highest concentration (10 μM), the oxidative metabolites and equol almost doubled the lag phase duration whereas FORM increased it by only 20%. In contrast to the ORAC assay, QUER and ASC exhibited comparable antioxidant capacities. Only DAI, FORM, BCA, PRU, and DHG showed lower antioxidant activities as ASC and QUER.

With respect to the effects of the test compounds on the duration of the lag phase by the formation of TBARS in the in vitro oxidation of LDL, again, the oxidative metabolites of DAI

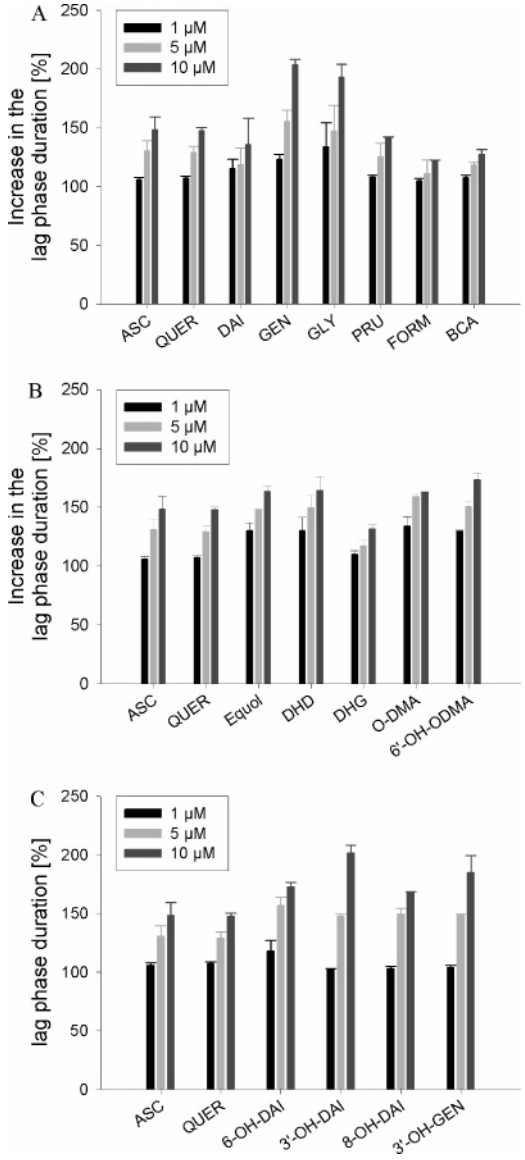

Figure 4. Effects of the naturally occurring isoflavones (**A**) and their respective bacterial (**B**) and oxidative (**C**) metabolites as compared to QUER and ASC, at concentrations of 1, 5, and 10 μM, on the duration of the lag phase during formation of conjugated dienes in the copper-induced in vitro oxidation of LDL. Bars show means ± standard deviations of at least triplicate experiments.

and GEN as well as equol are most effective (see **Figures 6** and **7**). However, in this assay, both QUER and ASC exhibited high antioxidant efficacy. Only 3′-OH-GEN and 8-OH-DAI are more effective than QUER and 3′-OH-GEN as well as 3′-, 6-, and 8-OH-DAI more effective than ASC. FORM showed once more the lowest antioxidant activity. As depicted in **Figures 6** and **7**, concentrations as low as 0.1 μM 3′-OH-GEN, 3′-, 6-, and 8-OH-DAI, and QUER are able to increase the lag phase duration by more than 30%. As already mentioned for the formation of conjugated dienes in the in vitro oxidation of LDL, a good correlation between antioxidant effect and concentration for all compounds was obtained.

DISCUSSION

Despite the various reports linking many of the beneficial properties of isoflavones (e.g., prevention of coronary heart disease as well as breast, prostate, and colon cancer) to their

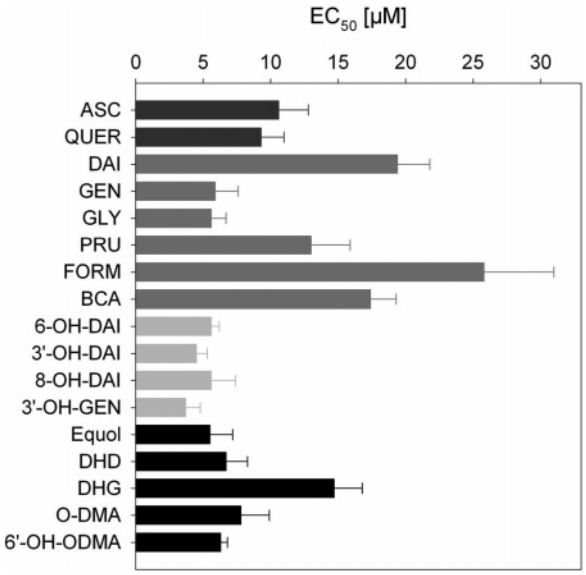

Figure 5. EC_{50} values (μM) for the tested isoflavones and their corresponding oxidative and bacterial metabolites as compared to QUER and ASC determined with the formation of conjugated dienes in the copper-induced in vitro oxidation of LDL. Bars show means ± standard deviations of at least triplicate experiments.

antioxidant properties, no comprehensive studies have been conducted comparing the antioxidant efficacy of the naturally occurring isoflavones with that of their metabolites. The aim of this study was to determine the antioxidant potencies of the naturally occurring isoflavones and their corresponding metabolites in order to establish structural features, which enhance antioxidant activities, and to identify the underlying mechanisms of the antioxidant properties.

In the ORAC assay, the ability of compounds to scavenge peroxyl radicals is assessed. In the in vitro oxidation of LDL scavenging of lipid peroxyl radicals, chelation of copper ions as well as stabilization of the LDL structure through interaction with apolipoprotein B to prevent binding of copper ions to the particles play a role in the prevention of lipid peroxidation (*11*, *12*). The inhibitory potencies of the test compounds were dependent on the system used. However, general rules underlying the structure/antioxidant activity relationship can be deduced. Specific structural criteria defining the free radical scavenging activities of flavonoids have already been characterized by Bors et al. (*13*). These include the 2,3-double bond with the 4-oxo group and the 3-hydroxyl group in the C-ring, the 5,7-dihydroxyl structure in the A-ring, and the ortho-dihydroxyl structure in the B-ring. The experiments conducted in this study also underline the contribution of the 5,7-dihydroxyl group in the A-ring since in all three assays GEN is more effective than DAI, which lacks the 5-hydroxyl group, and PRU, where the 7-hydroxyl group is blocked through methylation. *ortho*-Dihydroxyl structures whether in the A- or B-ring increase antioxidant efficacy since all oxidative metabolites of GEN and DAI exhibit higher antioxidant properties than the parent compounds. Furthermore, blocking hydroxyl groups through methylation, as for GLY as compared with 6-OH-DAI, BCA with GEN, FORM with DAI, and PRU with GEN, reduces the antioxidant capacities underlying the importance of phenolic hydroxyl groups for the antioxidant actions of isoflavones. Blocking the 4′-hydroxyl group as in the case of BCA and FORM almost abolished their antioxidant activity whereas blocking the hydroxyl group in the 7- or 6-position only reduces the antioxidant capacity slightly. Reduction of the isoflavone

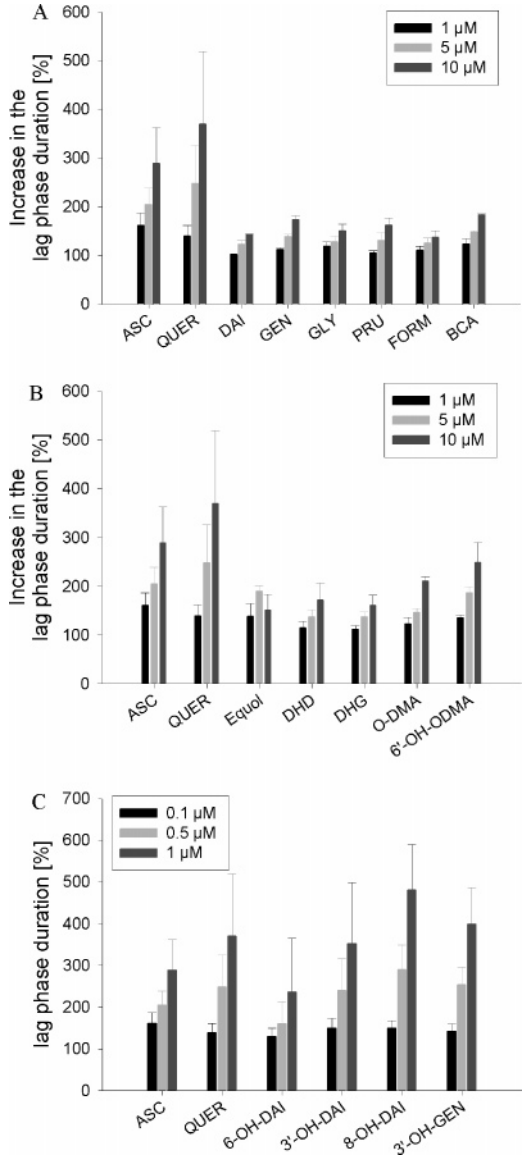

Figure 6. Effects of the naturally occurring isoflavones (**A**) and their respective bacterial (**B**) and oxidative (**C**) metabolites as compared to QUER and ASC on the duration of the lag phase during TBARS formation in the copper-induced in vitro oxidation of LDL. Concentrations used are as depicted. For ASC, 1, 5, and 10 μM were used, and for QUER, 0.1, 0.5, and 1 μM were used. Bars show means ± standard deviations of at least triplicate experiments.

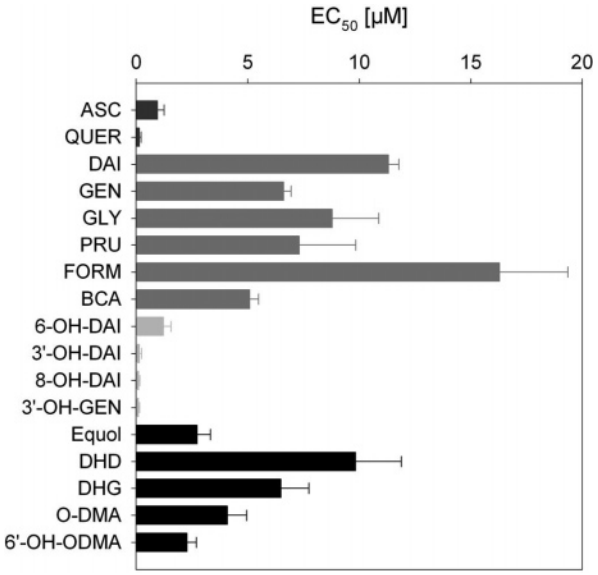

Figure 7. EC_{50} values (μM) for the tested isoflavones and their corresponding oxidative and bacterial metabolites as compared to QUER and ASC as determined with the TBARS formation in the copper-induced in vitro oxidation of LDL. Bars show means ± standard deviations of at least triplicate experiments.

molecule during the bacterial metabolism as for DHD, DHG, O-DMA, and 6′-OH-ODMA leads to the same or slightly lower antioxidant potencies. An exception is equol, which exhibits a higher antioxidant activity than DAI and about the same antioxidant capacity as the oxidative metabolites of DAI and GEN despite the lack of the 2,3-double bond with the 4-oxo group and a 5,7-diydroxyl structure. The reasons are still unclear. One might speculate that the nonplanar structure enables the molecule to penetrate more easily into protein or lipid structures and to prevent oxidative damage in situ.

Our findings with respect to the structure/antioxidant activity relationship are in agreement with the literature. There are several studies on the antioxidant properties of the well-established isoflavones DAI and GEN as well as of selected further naturally occurring isoflavones and metabolites, e.g., equol, BCA, or 8-OH-DAI. However, none of these studies have investigated the whole range of isoflavone metabolites and naturally occurring isoflavones (*14*−*21*). Several groups could show in agreement with our results that methylation of the hydroxyl group at C-4′ and conjugation with glucose or sulfate at the hydroxyl group at C-7 reduces the antioxidant capacity of isoflavones (*14*−*16*, *20*−*22*). In this study, we used PRU as an example where the hydroxyl group at C-7 is blocked by methylation instead of conjugation with glucose or sulfate. PRU was chosen in order to ensure a minimum of lipophilicity for the determination of the antioxidant activity in the in vitro oxidation of LDL. Furthermore, other groups could also show the outstanding antioxidant capacity of both equol and 8-OH-DAI (*15*−*18*).

As already mentioned above, the ORAC assay determines the ability of compounds to scavenge peroxyl radicals. Besides this ability, further antioxidant mechanisms necessary for acting as an effective antioxidant in the in vitro oxidation of LDL are chelation of copper ions as well as stabilization of the LDL structure through interaction with apolipoprotein B in order to prevent binding of copper ions to the particles and, thus, lipid peroxidation. The latter has already been shown to be a possible mechanism in the antioxidant action of ASC as well as in that of GEN, DAI, and equol (*11*, *23*−*25*). Furthermore, isoflavones are able to chelate metal ions (*26*, *27*). The current study shows that isoflavones and their metabolites are able to efficiently scavenge peroxyl radicals in a hydrophilic environment and to act as inhibitors of lipid peroxidation through different mechanisms in a lipophilic environment. This is possible since it has been demonstrated that isoflavones can be incorporated into LDL (*28*). However, the lower concentrations needed to increase the lag phase by 50% in the TBARS formation in the in vitro oxidation of LDL are striking. One might speculate that isoflavones show more pronounced radical scavenging properties than metal ion chelation or LDL stabilizing abilities since the formation of TBARS is a radical-driven reaction (*29*).

In conclusion, the current data on the antioxidant properties of naturally occurring isoflavones and their corresponding metabolites indicate that the metabolism of GEN and DAI to the oxidative metabolites 3′-OH-GEN and 3′-, 6-, and 8-OH-DAI as well as to the bacterial metabolite equol enhance their

antioxidant properties. Those compounds are more effective than the known antioxidants QUER and ASC. Because the efficacy of isoflavones as effective antioxidants is evident at concentrations well within the range found in the plasma of subjects consuming soy products, this biological activity could be of physiological relevance. However, demonstration of in vivo activity will require replication in a biological system.

ABBREVIATIONS USED

ASC, ascorbic acid; BCA, biochanin A; DAI, daidzein; DHD, dihydrodaidzein; DHG, dihydrogenistein; FORM, formononetin; GEN, genistein; GLY, glycitein; LDL, low-density lipoprotein; 6-OH-DAI, 6-hydroxy-daidzein; 8-OH-DAI, 8-hydroxy-daidzein; 3′-OH-DAI, 3′-hydroxy-daidzein; 3′-OH-GEN, 3′-hydroxy-genistein; 6′-OH-ODMA, 6′-hydroxy-*O*-desmethylangolensin; O-DMA, *O*-desmethylangolensin; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; PE, R-phycoerythrin; PRU, prunetin; QUER, quercetin; TBARS, thiobarbituric acid reacting substances.

LITERATURE CITED

(1) Nakamura, Y.; Tsuji, S.; Tonogai, Y. Determination of the levels of isoflavonoids in soybeans and soy-derived foods and estimation of isoflavonoids in the Japanese daily diet. *J. AOAC Int.* **2000**, *83*, 635−650.

(2) Klejdus, B.; Vitamvasova-Sterbova, D.; Kuban, V. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography−mass spectrometry after two-dimensional solid-phase extraction. *Anal. Chim. Acta* **2001**, *450*, 81−97.

(3) Chang, Y. C.; Nair, M. G. Metabolism of daidzein and genistein by intestinal bacteria. *J. Nat. Prod.* **1995**, *58*, 1892−1896.

(4) Joannou, G. E.; Kelly, G. E.; Reeder, A. Y.; Waring, M.; Nelson, C. A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *J. Steroid Biochem. Mol. Biol.* **1995**, *54*, 167−184.

(5) Heinonen, S. M.; Hikkala, A.; Wahala, K.; Adlercreutz, H. Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 285−299.

(6) Kulling, S. E.; Honig, D. M.; Simat, T. J.; Metzler, M. Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. *J. Agric. Food Chem.* **2000**, *48*, 4963−4972.

(7) Kulling, S. E.; Honig, D.; Metzler, M. Oxidative metabolism of the soy isoflavones daidzein and genistein in vitro and in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3024−3033.

(8) Cao, G.; Prior, R. L. Measurement of oxygen radical absorbance capacity in biological samples. *Methods Enzymol.* **1999**, *299*, 50−62.

(9) Kleinveld, H. A.; Hak-Lemmers, H. L.; Stalenhoef, A. F.; Demacker, P. N. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: Application of a short procedure for isolating low-density lipoprotein. *Clin. Chem.* ***1992***, *38*, 2066−2072.

(10) Ramos, P.; Gieseg, S. G.; Schuster, B.; Esterbauer, H. Effect of temperature and phase transition on oxidation resistance of low-density lipoprotein. *J. Lipid Res.* **1995**, *36*, 2113−2128.

(11) Gieseg, S. P.; Esterbauer, H. Low density lipoprotein is saturable by prooxidant copper. *FEBS Lett.* **1994**, *343*, 188−194.

(12) Esterbauer, H.; Ramos, P. Chemistry and pathophysiology of oxidation of LDL. *Rev. Physiol. Biochem. Pharmacol.* **1995**, *127*, 31−64.

(13) Bors, W.; Heller, W.; Michel, C.; Saran, M. Flavonoids as antioxidants: Determination of radical scavenging efficacies. *Methods Enzymol.* **1990**, *186*, 343−381.

(14) Ruiz-Larrea, M. B.; Mohan, A. R.; Paganga, G.; Miller, N. J.; Bolwell, G. P.; Rice-Evans, C. A. Antioxidant activity of phytoestrogenic isoflavones. *Free Radic. Res.* **1997**, *26*, 63−70.

(15) Arora, A.; Nair, M. G.; Strasburg, G. M. Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch. Biochem. Biophys.* **1998**, *356*, 133−141.

(16) Mitchell, J. H.; Gardner, P. T.; McPhail, D. B.; Morrice, P. C.; Collins, A. R.; Duthie, G. G. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. *Arch. Biochem. Biophys.* **1998**, *360*, 142−148.

(17) Rimbach, G.; de Pascual-Teresa, S.; Ewins, B. A.; Matsugo, S.; Uchida, Y.; Minihane, A. M.; Turner, R.; Vafeiadou, K.; Weinberg, P. D. Antioxidant and free radical scavenging activity of isoflavone metabolites. *Xenobiotica* **2003**, *33*, 913−925.

(18) Turner, R.; Baron, T.; Wolffram, S.; Minihane, A. M.; Cassidy, A.; Rimbach, G.; Weinberg, P. D. Effect of circulating forms of soy isoflavones on the oxidation of low density lipoprotein. *Free Radic. Res.* **2004**, *38*, 209−216.

(19) Hirota, A.; Inaba, M.; Chen, Y. C.; Abe, N.; Taki, S.; Yano, M.; Kawaii, S. Isolation of 8-hydroxyglycitein and 6-hydroxidaidzein from soybean miso. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 1372−1374.

(20) Arteaga, E.; Villaseca, P.; Rojas, A.; Marshall, G.; Bianchi, M. Phytoestrogens possess a weak antioxidant activity on low density lipoprotein in contrast to the flavonoid quercetin in vitro in postmenopausal women. *Climacteric* **2004**, *7*, 397−403.

(21) Chung, H. L.; Yang, L.; Xu, J. Z.; Yeung, S. Y. V.; Huang, Y.; Chen, Z. Y. Relative antioxidant activity of soybean isoflavones and their glycosides. *Food Chem.* **2005**, *90*, 735−741.

(22) Rimbach, G.; Weinberg, P. D.; de Pascual-Teresa, S.; Alonso, M. G.; Ewins, B. A.; Turner, R.; Minihane, A. M.; Botting, N.; Fairley, B.; Matsugo, S.; Uchida, Y.; Cassidy, A. Sulfation of genistein alters its antioxidant properties and its effect on platelet aggregation and monocyte and endothelial function. *Biochim. Biophys. Acta* **2004**, *1670*, 229−237.

(23) Retsky, K. L.; Freeman, M. W.; Frei, B. Ascorbic acid oxidation product(s) protect human low-density lipoprotein against atherogenic modification. Anti- rather than pro-oxidant activity of vitamin C in the presence of transition metal ions. *J. Biol. Chem.* **1993**, *268*, 1304−1309.

(24) Hwang, J.; Sevanian, A.; Hodis, H. N.; Ursini, F. Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid. *Free Radic. Biol. Med.* **2000**, *29*, 79−89.

(25) Ferretti, G.; Bacchetti, T.; Menanno, F.; Curatola, G. Effect of genistein against copper-induced lipid peroxidation of human high density lipoproteins (HDL). *Atherosclerosis* **2004**, *172*, 55−61.

(26) Record, I. R.; Dreosti, I. E.; McInerney, J. K. The antioxidant activity of genistein in vitro. *Nutr. Biochem.* **1995**, *6*, 481−485.

(27) Harper, A.; Kerr, D. J.; Gescher, A.; Chipman, J. K. Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. *Free Radic. Res.* **1999**, *31*, 149−160.

(28) Meng, Q. H.; Lewis, P.; Wahala, K.; Adlercreutz, H.; Tikkanen, M. J. Incorporation of esterified soybean isoflavones with antioxidant activity into low density lipoprotein. *Biochim. Biophys. Acta* **1999**, *1438*, 69−76.

(29) Janero, D. R. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **1990**, *9*, 515−540.

Received for review December 13, 2005. Accepted February 16, 2006. Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

JF053112O