Absorption and Excretion of the 8-Hydroxydaidzein in Rats after Oral Administration and Its Antioxidant Effect

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(Received July 13, 2004)

Summary 8-Hydroxydaidzein (8-OHD), which is produced during the processing of fermented soybean products, has a potent antioxidant activity in vitro. There is no information regarding the absorption and excretion of this isoflavone, including its antioxidant effect in vivo. In this study, rats were administered a single oral dose of 8-OHD (20 mg/kg body weight), and the blood, liver, kidney and urine were collected at specific intervals up to 18 h after dosing. Free 8-OHD in each tissue was directly determined by using HPLC with electrochemical detection, while its conjugates were detected after the treatment with β-glucuronidase and sulfatase. The total 8-OHD in liver reached a high level (9.4 nmol/g) at 1 h after dosing, and maintained the relatively high concentration up to 10 h. Most of the 8-OHD was present in free form in liver, while the majority of 8-OHD in plasma was conjugated. This suggests that free 8-OHD in liver is successively converted to glucuronide and/or sulfate and the conjugated 8-OHD is released into the blood. The maximum level of total 8-OHD in plasma or kidney was observed within the first 2 h after the oral administration. The level of 8-OHD in these tissues gradually decreased within the further experiments. Excretion of the 8-OHD in urine began to rise at 1–2 h interval. The mean urinary excretion rate of 8-OHD showed a higher level at 2–4 h and 4–6 h intervals, while the 8-OHD levels at these intervals in plasma or kidney more rapidly decreased. The cumulative recovery of 8-OHD in the urine over the 0–18 h interval was about 36% of the dose. In addition, the liver homogenate from rats killed at 1 h and 2 h after dosing, which contained a higher level of free 8-OHD, showed a significantly lower susceptibility to lipid peroxidation induced by AAPH or Cu²⁺ than that at 0 h (pre-administered rats). These results suggest that 8-OHD was relatively easily absorbed into rats and might exert its biological activities in vivo, including the antioxidant effect.

Key Words 8-hydroxydaidzein, fermented soy products, antioxidant, absorption and excretion, rats

The isoflavonoid phytoestrogens, found in numerous plants, especially soybeans, are associated with a broad variety of properties beneficial to human health. Epidemiological studies have shown that the consumption of soybeans and soy products is effective for reducing the risk of hormone-related tumors, such as breast and prostate cancer, as well as osteoporosis and coronary heart disease (1–3). Daidzein and genistein, the representative soy isoflavone aglycones, have an antioxidant potential, and the intake of them in humans promoted the resistance of low density lipoprotein against oxidation (4). Genistein is also a potent and specific inhibitor of protein tyrosine kinases (5), many of which form part of growth factor-stimulated signal transduction cascades in normal and transformed cancer cells (6). These findings suggest that isoflavones in soybean foods might play an important role in human health maintenance.

In East Asian countries including Japan, there are many traditional fermented products using soybeans. In soybeans as well as in non-fermented soy products, the isoflavones mainly occur in glucosides (daidzin and genistin) and their esterified forms (malonylglucosides and acetylglucosides), whereas in fermented soy products such as soybean pastes (miso) and soy sauces (shoyu) their aglycones (daidzein and genistein) predominate (7). During the manufacturing of these fermented products, the isoflavone glucosides in the heated soybean original were hydrolyzed to liberate their corresponding aglycones by β-glucosidase produced from Aspergillus species in the koji making process followed by fermentation/ripening periods (8, 9). Recently, we have found potent new antioxidative isofla-
vones from soybean miso (mame miso) and soy sauces (shoyu and tamari), in addition to their koji (10). These isoflavones, 8-hydroxydaidzein (8-OHD) and 8-hydroxygenistein (8-OHG), which have an o-dihydroxy structure between the 7- and 8-position, were produced from daidzein and genistein, respectively, by a hydroxylation reaction during the fermentation process (9, 11). In the case of soybean miso, the amounts of 8-OHD and 8-OHG were 5.1 mg and 9.8 mg per 100 g of paste, respectively, which corresponded to 10 wt% and 15 wt% of daidzein and genistein, respectively (12). 8-OHD and 8-OHG respectively showed about 9 times and 11 times stronger antioxidative activities in comparison with daidzein and genistein in a lipid/aqueous system using a liposome (13). According to these facts, o-dihydroxyisoflavones (ODI) might contribute to protection from oxidative deterioration during the processing and storing of these soybean products.

In the last few years, studies have focussed on the absorption, distribution and metabolism or excretion of soy isoflavones. It is generally known that, following ingestion, the isoflavone glucosides are converted to the corresponding aglycones by gut microflora or gut glucosidases, and then the aglycones are absorbed from the small intestine (14, 15). In addition, it is also reported that the isoflavone aglycones were absorbed faster and in greater amounts than their glucosides in humans (16). This result shows that the isoflavone aglycone-rich fermented soy products such as soybean miso may be more effective than glucoside-rich products in preventing various lifestyle diseases, especially hormone-related diseases. In this study, we investigated the absorption and excretion of this potent antioxidative 8-OHD in rats after its oral administration. In addition, we examined the antioxidative effect on susceptibility of the liver homogenate of these rats to the lipid peroxidation.

**MATERIALS AND METHODS**

**Materials.** Chemicals of analytical grade were used in this study. β-Glucuronidase/sulfatase (Type H-2 from Helix Pomatia, G 0876) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 8-OHD was prepared from soy sauce (Tamari) cake using an Amberlite XAD-2 column followed by preparative high-performance liquid chromatography (HPLC) (13, 17). The identification and purity of 8-OHD were confirmed by three-dimensional HPLC equipped with a photodiode array before use (13).

**Animals and diets.** Five week-old male Sprague-Dawley (SD) rats (n=48) weighing 130-150 g were obtained from Japan S.L.C. (Hamamatsu, Japan). The rats were housed in individual wire screen-bottomed cages at 25°C with a 12-h light-dark cycle and allowed free access to commercial chow (CA-1 pellet rations, CLEA Japan) and drinking water for 1 wk. The rats were then fed an isoflavone-free synthetic diet for 6d to allow elimination of circulating isoflavones likely to arise from the normal unpurified diet before being used in the experiments. Composition of the diet was as follows (g/kg): casein, 200.0; α-cornstarch, 529.5; sucrose, 100.0; cellulose powder, 50.0; corn oil, 70.0; AIN-93G mineral mix (18), 35.0; AIN-93 (VE-free) vitamin mix (18), 10.0; L-cysteine, 3.0; and choline bitartrate 2.5. All procedures were performed in accordance with the Animal Experimentation Guides of Nagoya University.

**Experimental design and sample collection.** The rats were transferred to metabolism cages and fasted for 24 h. Urine was collected into Erlenmeyer flasks containing 10 mg of ascorbic acid for 24 h to provide pretreatment samples. 8-OHD, which was dissolved in 70% glycerol, was administered at 6 p.m. as an oral dose by stomach tube (20 mg 8-OHD/kg body weight), except for the pre-administered group (n=6). Six rats were anesthetized with ethyl ether at 0 (pre-administered rat), 1, 2, 4, 6, 10, 14 and 18 h after 8-OHD dosing, and the blood was collected by cardiac puncture into a heparinized syringe. The plasma was immediately prepared by centrifugation at 1,600 ×g for 15 min at 4°C and then stored at −80°C until assayed. The liver, which was perfused with physiological saline, and kidneys were excised from the rats after blood collection. A section of each of them was kept at −80°C until assayed. Urine for isoflavone measurement was also collected for the intervals 0–1, 1–2, 2–4, 4–6, 6–10, 10–14 and 14–18 h after 8-OHD dosing. The urine collected at different periods was lyophilized individually. Each dried material was dissolved in 1.5 mL of water and analyzed.

**Determination of 8-OHD in plasma and urine.** Free and conjugated isoflavones were determined by HPLC equipped with an electrochemical detector (HPLC-ECD) after extraction from non-enzyme and enzyme treated samples. Methanol was used for the extraction of 8-OHD in plasma and urine (19). For determination of free 8-OHD, triplicate 0.1 mL aliquots of plasma or urine were mixed with 0.4 mL of 0.1 M sodium acetate buffer (pH 5.0) and 1.5 mL of methanol. On the other hand, the conjugated 8-OHD in plasma or urine was hydrolyzed by the mixture of β-glucuronidase and sulfatase (Type H-2). For determination of total 8-OHD, triplicate 0.1 mL aliquots of plasma or urine were treated with β-glucuronidase (500 units) and sulfatase (12 units) dissolved in the same buffer (0.4 mL) for 60 min at 37°C, and then the same volume (1.5 mL) of methanol was added. The enzyme-treated and non-treated mixtures were vortex-mixed for 30 s, sonicated 30 s and finally centrifuged at 5,000 ×g for 5 min at 4°C. The supernatant and/or diluted sample (10 μL) was injected onto an HPLC column (Develosil ODS-UG-5, 2.0 i.d. × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan). The column temperature was kept at 40°C. A mixture of methanol and 0.1 mM phosphate buffer (pH 5.5) (35:65, v/v) containing 5 ppm EDTA was used as a mobile phase at a flow rate of 0.2 mL/min. The eluate was monitored with an electrochemical detector (ECD 3005, Shiseido, Tokyo, Japan) operating at a potential of 0.7 V. Quantification of 8-OHD was done by measuring the peak areas based on calibration plots of the peak area of standard 8-OHD at various concentrations. Preliminary studies showed that 98.5±3.4% of an 8-OHD spike
was recovered from plasma and 88.9±5.3% from urine by this method. The results reported here have not been corrected for these losses. In addition, the above supernatant was also employed in three-dimensional HPLC analysis (Develosil ODS-UG-5, 4.6 i.d.×250 mm, Nomura Chemical Co., Ltd., methanol–water (35:65, v/v) containing 0.1% (v/v) trifluoroacetic acid, 0.7 mL/min. photodiode array detector (SPD-M10A, Shimadzu)) for the identification of 8-OHD.

**Determination of 8-OHD in liver and kidney.** A section from the liver or kidney (2.0 g) was homogenized with 6 mL of cold 0.1 M sodium acetate buffer (pH 5.0) in a homogenizer with a Teflon pestle on ice. For determination of free 8-OHD, triplicate 0.6 mL aliquots of the tissue homogenates were mixed with 0.6 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 2.0% ascorbic acid and 0.01% EDTA. On the other hand, triplicate 0.6 mL aliquots of the same tissue homogenates were treated with β-glucuronidase (3,000 units) and sulfatase (72 units) dissolved in the same buffer (0.6 mL) for 60 min at 37°C to determine the total 8-OHD. Three milliliters of ethyl acetate was added to both the enzyme-treated and non-treated preparations. The mixtures were vortex-mixed for 30 s and sonicated for 30 s. Ethyl acetate layer was recovered by centrifugation at 1,600 × g for 5 min at 4°C, and then dried with anhydrous sodium sulfate. The aliquots (1.0 mL) were evaporated to dryness under reduced pressure. The residue was dissolved in 5.0 mL of methanol, and the resulting aliquot (10 μL) was used for the determination of 8-OHD by HPLC-ECD under the same conditions as described above. Preliminary studies showed that 85.0±5.5% of an 8-OHD spike was recovered from liver homogenate and 91.8±3.1% from kidney sample by this method. In addition, the above concentrated methanol solution was also analyzed by three-dimensional HPLC for the identification of 8-OHD.

**Measurement of oxidative susceptibility of liver homogenate.** The liver obtained from 3 representative rats, which were killed at 0, 1, 2, 4, 6, 10, 14 and 18 h after 8-OHD dosing, was used to examine the liver oxidative status. Each frozen liver (0.5 g) was homogenized in 9 vol of 20 mM phosphate buffer (pH 7.4). Oxidation resistance of the liver homogenate was measured according to the method of Tsuda et al. (20), using the 2,2′-azobisis (2-amidinopropane) dihydrochloride (AAPH) and copper sulfate (Cu²⁺). The triplicate 0.9 mL aliquots of each liver homogenate were mixed with 0.1 mL of 100 mM AAPH or 1 mM Cu²⁺, and the mixtures were incubated at 37°C in light-shaded test tubes. After the oxidation at specific intervals, 0.1 mL of 2% butylhydroxytoluene was added to the reaction mixtures, and the TBARS was determined by the method of Naito and Yamanaka (21).

**Statistical analyses.** The data were expressed as the means±SD. The results were submitted to a one-way analysis of variance (ANOVA) and to Dunnett’s test. The differences where p<0.05 were considered to be statistically significant.

**RESULTS**

**Quantitative changes of 8-OHD in plasma, liver and kidney**

Standard 8-OHD was detected as the peak eluted at 11 min by HPLC-ECD, and then the peak exhibited absorption maximum at 258 nm in UV spectrum by three-dimensional HPLC analysis. In plasma, liver and kidney, no free or conjugated isoflavones including 8-OHD were detected before an oral administration of 8-OHD (pre-administered rat). After an oral ingestion of 8-OHD, the peak of 8-OHD appeared in β-glucuronidase/sulfatase treated and non-treated samples from plasma, liver and kidney. The peak observed by the HPLC-ECD was further identified as the 8-OHD by three-dimensional HPLC. The photodiode array spectrum of the 8-OHD peak obtained from each sample was completely identical to that of the standard.

Time-course changes in free 8-OHD and total 8-OHD (free and conjugated forms) concentrations in liver following an oral administration (20 mg/kg body weight) of 8-OHD. Values are the mean±SD of six rats at each time point.

![Figure 1: Time-course changes in free 8-OHD and total 8-OHD (free and conjugated forms) concentrations in liver following an oral administration (20 mg/kg body weight) of 8-OHD. Values are the mean±SD of six rats at each time point.](image-url)
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Fig. 2 Time-course changes in free 8-OHD and total 8-OHD (free and conjugated forms) concentrations in plasma following an oral administration (20 mg/kg body weight) of 8-OHD. Values are the mean±SD of six rats at each time point.

Fig. 3 Time-course changes in free 8-OHD and total 8-OHD (free and conjugated forms) concentrations in kidney following an oral administration (20 mg/kg body weight) of 8-OHD. Values are the mean±SD of six rats at each time point.

shown in Fig. 3. Both the free and the total 8-OHD reached higher concentrations within the first 2 h after the administration. The free 8-OHD levels at 1 and 2 h were 24.2 and 25.4 nmol/g, respectively. There was no significant difference between these values. The concentrations of total 8-OHD at 1 and 2 h were 39.1 and 36.4 nmol/g, respectively. The levels of free and total 8-OHD gradually decreased in the same way as liver and plasma. Free 8-OHD comprised nearly 60% of the total present in kidney at 1 h, and this share continued to 10 h.

Urinary excretion of 8-OHD following the oral administration

Urinary excretion rates of free and conjugated 8-OHD at the different collecting periods following an oral administration of 8-OHD are shown in Fig. 4. In the pre-administered group (Pre), no free or conjugated 8-OHD was detected. Both the free and the conjugated 8-OHD were detected in all periods following the oral administration. The excretion rate of free 8-OHD at 0–1 h was very slight (0.08 μmol/h). However, it increased to a maximum (0.22 μmol/h) in the 2–4 h interval and almost maintained a plateau until 6–10 h. In the conjugated form of 8-OHD, the excretion rate also increased after the administration and showed a maximum (0.36 μmol/h) at 2–4 h. Then, the rate declined during the subsequent periods. The excretion percentages of the free form in total 8-OHD was nearly 30–60% throughout the urine collection periods. In Fig. 5, cumulative urinary excretions of the total and the free 8-OHD following an oral administration of 8-OHD are illustrated. The cumulative level of total 8-OHD increased with the progress in the time after the administration and reached a high (6.11 μmol) after 18 h. The free form of 8-OHD excreted in the urine also gradually increased, and the level after 18 h was 2.82 μmol.

Susceptibility of liver homogenate to lipid peroxidation

The TBARS levels generated in each liver homogenate exposed to AAPH during 0 h (white bar) and 4 h (black bar) are shown in Fig. 6A. Before the oxidation by AAPH (0 h), the TBARS levels in the liver killed at 0, 1, 2, 4, 6, 10, 14 and 18 h were in the range of 32.5 to
Several active oxygen species such as the superoxide anion radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen, including lipid hydroperoxide and its related radicals, are known to cause some oxidative damages in living systems. Dietary antioxidants are supposed to protect cells and tissues against damage caused by these active oxygen species. Soybeans also contain many kinds of antioxidants such as isoflavones, tocopherols, saponins, etc. Recently, the studies on absorption, accumulation, metabolism and excretion of these phytochemicals after oral ingestion have attracted much attention (22-24). It is very important to evaluate the bioavailability of food antioxidants, and then to clarify whether the ingested dietary ingredients function as antioxidants in vivo.

In this study, the potent antioxidative 8-OHD was administered orally to SD rats, and firstly the concentrations of free 8-OHD and its conjugates (glucuronide and/or sulfate) in rat liver, plasma, kidney and urine were monitored after the administration. The conjugated isoflavones were hydrolyzed with β-glucuronidase/sulfatase and then the total 8-OHD concentration was determined. When rats were given 8-OHD in 70% glycerol (74 μmol/kg body weight) orally, the total 8-OHD in liver attained a higher concentration (9.4 nmol/g liver) at 1 h after the oral intake, then gradually decreased (Fig. 1). However, a relatively high level of 8-OHD was maintained until 10 h. It is also noteworthy that most 8-OHD in liver existed in the free form, not in the conjugated type, because o-dihydroxy groups at the 7- and 8-positions of isoflavone skeleton play an important role in antioxidant reaction. Miyazawa et al. (24) reported that the level of free cyanidin-3-glucoside reached a maximum at 15 min after the oral intake in rat liver and plasma. The time to reach maximal concentration in liver and plasma was shorter in anthocyanidins than that in tea catechin, epigallocatechin gallate (30 min). These results, including the time to reach the maximum in 8-OHD, may indicate that water-soluble substances reach a rapid maximum level after their oral administration.

King et al. (25) have studied the pharmacokinetics of the soy isoflavone genistein following the single oral dose (20 mg/kg body weight, freshly prepared in 25 mm sodium carbonate) in rats. The plasma was treated with β-glucuronidase and the total genistein was measured. The genistein concentration in plasma reached a maximum level (11.0±2.3 nmol/mL) at 2 h after oral dosing. In the another report (26), rats were given a single oral dose of a soy extract (prepared in sodium carbonate) that provided 74 μmol (20 mg) genistein and 77 μmol (19.6 mg) daidzein/kg body weight (as glycosidic conjugates). The plasma daidzein concentration was maximal at 2 h (9.5±0.71 nmol/mL) and was almost double that of genistein. In this study, 8-OHD was dissolved in 70% glycerol and administered to rats (20 mg/kg body weight). At 1 h after dosing, the total concentration of 8-OHD in plasma was 6.9±3.9 nmol/mL (Fig. 2). The 8-OHD concentration further increased, and the level at 2 h was 8.2±1.1 nmol/mL. The 8-OHD maximal concentration in plasma was about the same level compared with that of daidzein. Uehara et al. (27) have reported the influence of dietary fructooligosaccharides on bioavailability of genistein and daidzein in rats. A single dose of soy isoflavone conjugates (suspended in water), i.e., 8.5 mg as genistein and 33 mg as daidzein/kg body weight, was administered via stomach tube. In the control group, the daidzein concentration in the central venous blood reached peaks at 3 and 6 h (2.5±0.6 and 2.5±0.7 nmol/mL, respectively) and declined thereafter. The variability among results of these studies may be due in part to the use of different doses and forms of administration, but also to differences in the conditions of administered substances (i.e., dissolved or suspended) (23).

In plasma, most 8-OHD was detected in the conjugated form. This result suggests that the free form of 8-OHD in liver may be conjugated under hepatic gluco-
ronization and/or sulfation reaction (28) and the conjugated 8-OHD released into the blood. 8-OHD possesses three hydroxyl groups at the 4'-, 7- and 8-positions which are available for glucuronidation and/or sulfation. In future, it will be necessary to clarify which position of 8-OHD is used for the conjugation in order to evaluate its antioxidant potential. The free form of 8-OHD was also found in plasma. A small amount of free 8-OHD was more than twofold higher than that of 8-OHD administration was examined by using AAPH and detected after the oral administration (Fig. 3). The total was too low (at 1-10h, 0.7-1.5 nmol/mL) to exert antioxidant activity (13).

In kidney, a higher concentration of 8-OHD was detected after the oral administration (Fig. 3). The total 8-OHD reached higher levels within the first 2 h (at 1 h, 39.1±15.9 nmol/g kidney; at 2 h, 36.4±14.5 nmol/g kidney), then gradually decreased to 8.5±4.3 nmol/g at 10 h. The level of total 8-OHD at this time was almost consistent with that at 1 h in liver (Fig. 1). The free form of 8-OHD, which possesses potent antioxidant activity, also existed in relatively higher concentration in kidney. The percentage of free form in total 8-OHD was 60–70% during 1–10 h following the oral administration. These levels of free 8-OHD might be great advantage in improving the antioxidant status.

Urinary excretion following the oral administration was also studied in the present experiment. The mean excretion rate of the total 8-OHD per hour gradually increased during the first 4 h (Fig. 4). The maximal rate at 2–4 h was 0.58 μmol/h. This excretion rate of 8-OHD was more than twofold higher than that of genistein reported by King et al. (25). The cumulative level of total 8-OHD excretion in urine reached high (6.11μmol) after 18 h (Fig. 5). This value indicates that 36.0% of the administered 8-OHD was recovered after 18 h. This urinary recovery rate of 8-OHD in rats was greater than that (19.9%) of genistein, which was administered in aglycone form (25). When rats were given isoflavone conjugates (a soy extract), urinary excretion of daidzein over the 48 h postdose period was 17.4% of the dose, while 11.9% of the genistein dose was excreted in urine (26).

These findings suggest that in absorption and excretion, 8-OHD has a relatively shorter half-life than daidzein and genistein. Comparing the kinetics and bioavailability of 8-OHD with those of daidzein and genistein in rats, 8-OHD seemed to be absorbed considerably more rapidly than daidzein and genistein, and the relative bioavailability of 8-OHD compared to these isoflavones might be estimated to be about 2 or 3 times greater. Further investigations are required to confirm these experimental facts.

In the second study, oxidative susceptibility of the rat liver homogenate killed at different times following 8-OHD administration was examined by using AAPH and Cu2+ (Fig. 6A, B). There was not a great difference in the TBARS value among the liver before the oxidation by AAPH. When the lipid peroxidation was induced by AAPH, the TBARS values of liver homogenate at 1 and 2 h, which showed high levels of free 8-OHD (Fig. 1), were significantly lower than that from the 0 h sample. In the presence of Cu2+, the lipid peroxidation at 1 h was also significantly (p<0.05) suppressed compared with that from the 0 h sample. These results suggest that potent antioxidant 8-OHD absorbed into liver after the administration could increase the oxidation resistance of the liver.

This oxidative susceptibility of the plasma was also studied (data not shown), but the oxidation resistance was not appreciated because of the low level of free 8-OHD. Further studies concerning the oxidative susceptibility of the rat kidney should be conducted.

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