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Antioxidant Effects of Isorhamnetin 3,7-Di-*O*-β-D-glucopyranoside Isolated from Mustard Leaf (*Brassica juncea*) in Rats with Streptozotocin-Induced Diabetes

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To investigate the effects of isorhamnetin 3,7-di-*O*-β-D-glucopyranoside (isorhamnetin diglucoside), a major flavonoid compound of mustard leaf, on oxidative stress due to diabetes mellitus, in vivo and in vitro studies were carried out. Oral administration of isorhamnetin diglucoside (10 or 20 mg/kg of body weight/day for 10 days) to rats with streptozotocin-induced diabetes significantly reduced serum levels of glucose and 5-(hydroxymethyl)furfural (5-HMF), which is glycosylated with hemoglobin and is an indicator of oxidative stress. After intraperitoneal administration, isorhamnetin diglucoside did not show these activities. In addition, after oral administration, the thiobarbituric acid-reactive substance levels of serum, and liver and kidney mitochondria declined significantly compared with the control group in a dose-dependent manner, whereas after intraperitoneal administration these levels fell only slightly. On the basis of the oral and intraperitoneal results, it was hypothesized that isorhamnetin diglucoside was converted to its metabolite in vivo, and its conversion to its aglycone, isorhamnetin, by β-glucosidase was confirmed; isorhamnetin acted as an antioxidant. Moreover, it was observed that isorhamnetin diglucoside had no effect on the 1,1-diphenyl-2-picrylhydrazyl radical, whereas isorhamnetin showed a potent antioxidant effect in vitro. In addition, intraperitoneal administration of isorhamnetin reduced serum glucose and 5-HMF levels. Furthermore, lipid peroxidation in blood, liver, and kidney associated with diabetes mellitus declined after the administration of isorhamnetin. These results suggest that isorhamnetin diglucoside is metabolized in vivo by intestinal bacteria to isorhamnetin and that isorhamnetin plays an important role as an antioxidant.

KEYWORDS: Isorhamnetin 3,7-di-*O*-β-D-glucopyranoside; streptozotocin; 1,1-diphenyl-2-picrylhydrazyl radical; thiobarbituric acid-reactive substance; 5-(hydroxymethyl)furfural; diabetes

INTRODUCTION

Oxidative stress is linked to tissue damage and the development of pathophysiology and occurs in most, if not all, human diseases. In addition, oxidative stress and oxidative damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis. In particular, since the early 1980s, a role for reactive oxygen species in diabetes has been widely discussed, and there is considerable evidence that oxidative damage is increased in diabetes, although the mechanisms involved are not clear. Furthermore, there are reports that oxygen species are generated as a result of hyperglycemia and cause many of the secondary complications of diabetes, such as nephropathy, retinopathy, and neuropathy (*1*−*4*). Therefore, a wide range of antioxidants, both natural and synthetic, have been proposed for use in the treatment of human disease.

Recently attention has been focused on the antioxidative activities of many plant phenolics, especially flavonoids, because they inhibit lipid peroxidation, and the significance of potential protective properties of flavonoids present in vegetables and fruits has become an important issue (*5*, *6*). Flavonoids are diphenylpropanes occurring ubiquitously in food plants and are common components in the human diet. Several studies have demonstrated the physiological functions of flavonoids, such as hypotensive, antiallergic, anti-inflammatory, antiviral, anticancer, and anticarcinogenic properties (*7*−*9*). Mustard leaf (*Brassica juncea*), a major raw material for kimchi in Korea

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and one of the cruciferous vegetables well-known to contain high levels of flavonoids that have attracted much attention as antioxidants, is also expected to be an effective antioxidant due to these flavonoids. Although mustard leaf has been demonstrated to have the antiatherogenic effect of reducing plasma cholesterol levels and increasing high-density lipoprotein cholesterol levels (*10*), its antioxidant activities are unclear to date. In addition, other active compounds besides flavonoids in mustard leaf, thioglucosides, have been isolated (*11*). However, the major active flavonoid in which mustard leaf is rich and its metabolism in vivo have not been elucidated yet.

In this study, we investigated whether the main compound, isorhamnetin 3,7-di-*O*-β-D-glucopyranoside (isorhamnetin diglucoside), is useful in the improvement of diabetes caused by oxidative stress and also examined its metabolic pathway and the antioxidative activity of its metabolite.

MATERIALS AND METHODS

Purification of Isorhamnetin Diglucoside from Mustard Leaf. As described previously (*12*), dry leaves (14.8 kg) were refluxed with methanol (MeOH) for 3 h, and the MeOH extract (1600 g) was suspended in distilled H_2O and partitioned with CH_2Cl_2, ethyl acetate (EtOAc), *n*-butanol (n-BuOH), and H_2O in sequence, thus yielding four fractions (CH_2Cl_2, 304 g; EtOAc, 10 g; n-BuOH, 124 g; H_2O, 1140 g). Then, the BuOH fraction (124 g) was subjected to silica gel column chromatography. Elution with CH_2Cl_2 containing increasing amounts of MeOH (50, 10−20, and 30%) and then MeOH gave 11 subfractions. Subfraction 6 (41.2 g) was further purified by Sephadex LH-20 column chromatography using MeOH as a solvent to give isorhamnetin diglucoside (1800 mg).

Preparation of Isorhamnetin from Isorhamnetin Diglucoside. Isorhamnetin diglucoside (600 mg) was refluxed with 5% H_2SO_4 in MeOH (100 mL) at >80 °C for 3 h. The reaction mixture was cooled, concentrated to half its original volume, added to iced H_2O, and the precipitate was collected by filtration. Isorhamnetin (220 mg) was obtained after washing and drying the precipitate.

Reagents. Streptozotocin (STZ) and naringinase were purchased from Sigma Chemical Co. (St. Louis, MO). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animal Experiments. *(1) Animal Preparation.* Male Wistar rats (5 weeks old, 120−130 g) from Japan SLC Inc. (Hamamatsu, Japan) were used. They were kept in a wire-bottom cage under a conventional lighting regimen with a dark night. The room temperature (~25 °C) and humidity (~60%) were controlled automatically. Laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan, comprising 24.0% protein, 3.5% lipid, and 60.5% carbohydrate) and water were given ad libitum. After several days of adaptation, STZ (50 mg/kg of body weight) dissolved in citrate buffer (pH 4.5) was injected intraperitoneally following overnight fasting. One week after injection, the glucose level of blood taken from the tail vein of each rat was determined, and then the animals were divided into experimental groups. The control group was given physiological saline (vehicle), whereas the other groups were given isorhamnetin diglucoside (at a dose of 10 or 20 mg/kg of body weight/day) or isorhamnetin (at a dose of 10 mg/kg of body weight/day) orally or intraperitoneally. After 10 consecutive days of administration, the rats were killed by decapitation, the blood was collected, and the livers and kidneys were removed, rinsed with cold physiological saline, and frozen at −80 °C until assayed.

(2) Determination of Serum Glucose Level. The serum glucose level was measured using a commercial kit (Glucose CII-Test Wako; Wako Pure Chemical Industries).

(3) Determination of Serum Glycosylated Hemoglobin Level. Glycosylated hemoglobin level in serum was determined by the thiobarbituric acid (TBA) assay reported by Fluckiger and Winterhalter (*13*) with modification.

(4) Determination of Serum TBA-Reactive Substance Level. The TBA-reactive substance level in serum was determined using the method of Naito and Yamanaka (*14*).

(5) Preparation of Mitochondria and Measurement of TBA-Reactive Substance Level. Mitochondria from liver or kidney homogenates was prepared by differential centrifugation (800*g* or 12000*g*) with a refrigerated centrifuge (4 °C) using the methods of Johnson and Lardy (*15*) and Jung and Pergande (*16*) with slight modifications. Each pellet was resuspended with prepared medium, and the TBA-reactive substance level was determined according to the method of Uchiyama and Mihara (*17*).

(6) Determination of Protein. Protein content was quantified using a commercial protein assay kit (A/G B-Test Wako; Wako Pure Chemical Industries) or by the method of Itzhaki and Gill (*18*) with bovine serum albumin as the standard.

Determination of DPPH Radical Level. A 100 μL aliquot of an aqueous solution of the sample (control = 100 μL of distilled water) was added to microwells followed by an ethanolic solution of DPPH (final concentration = 60 μM), according to the method of Hatano et al. (*19*). After gentle mixing and 30 min of standing at room temperature, the DPPH radical level was measured with a microplate reader, model 3550-UV (Bio-Rad, Tokyo, Japan). The antioxidant activity was expressed as the IC_{50} (concentration in μg/mL required to inhibit DPPH radical formation by 50%) determined from the log−dose inhibition curve.

Analysis of Hydrolysates by HPLC. The hydrolysates of isorhamnetin diglucoside using HCl (pH 2) or β-glucosidase from naringinase were analyzed by HPLC. The HPLC system used consisted of a PX-8020, CCPM-II, and UV-8020 (Tosoh Co., Ltd.). The column used was an ODS-80 Ts analytical column (4.6 i.d. × 150 mm), the mobile phase consisted of a 20%/80% (by volume) mixture of 100% MeOH/2% acetic acid at a flow rate of 1 mL min^{-1}, and the UV detector was set at 265 nm.

Statistical Analysis. Data are presented as means ± SE. Differences among groups were analyzed by Dunnett's test, and those at $p < 0.05$ were considered to be significant.

RESULTS

Serum Glucose and Glycosylated Hemoglobin Levels. The STZ-induced diabetic rats had higher glucose and 5-HMF levels than normal rats, as shown in **Table 1**. However, the serum glucose levels of rats given 10 and 20 mg of isorhamnetin diglucoside orally were 510.1 and 492.4 mg/dL, respectively, whereas the control level was 574.2 mg/dL. Similarly, oral administration had a very significant and dose-dependent inhibitory effect on glycosylated hemoglobin formation by 28 and 43% decrease at the oral doses of 10 and 20 mg, respectively. In contrast, isorhamnetin diglucoside administered intraperitoneally did not change the serum glucose and 5-HMF levels in comparison with the controls.

TBA-Reactive Substance Levels in Serum and Hepatic and Renal Mitochondria. As shown in **Figure 1**, the diabetic rats showed higher lipid peroxidation of their serum, livers, and kidneys than normal rats. The serum TBA-reactive substance level of diabetic rats given isorhamnetin diglucoside orally was significantly lower than that of the diabetic control group, but intraperitoneal administration did not affect the TBA-reactive substance level. After an oral dose of 20 mg, the serum TBA-reactive substance level decreased by 57%. Similarly, the hepatic and renal mitochondrial levels of oral administration showed significant decreases compared with the control group levels, whereas after intraperitoneal administration, these levels fell only slightly. In the rats fed 20 mg orally, the TBA-reactive substance level of the hepatic mitochondria declined by 35% and that of the renal mitochondria fell by 38%, whereas after intraperitoneal administration, the TBA-reactive substance levels of hepatic and renal mitochondria showed smaller decreases by 12 and 16%, respectively.

Identification of Isorhamnetin from Isorhamnetin Diglucoside. Hydrolysis of isorhamnetin diglucoside with HCl (pH

Table 1. Effect of Isorhamnetin Diglucoside on Serum Glucose and 5-(Hydroxymethyl)furfural Levels after Oral or Intraperitoneal Administration

route	group	dose (mg/kg of BW/day)	glucose[a] (mg/dL)	5-HMF[a] (nmol/mg of protein)
oral	diabetic rats			
	control		574.2 ± 23.0a	21.12 ± 1.88a
	isorhamnetin diglucoside	10	510.1 ± 6.5ab	15.16 ± 0.75ab
	isorhamnetin diglucoside	20	492.4 ± 14.4ab	11.99 ± 1.01ab
intraperitoneal	diabetic rats			
	control		522.0 ± 20.2a	17.16 ± 0.27a
	isorhamnetin diglucoside	10	538.5 ± 11.4a	18.66 ± 0.31a
	isorhamnetin diglucoside	20	520.7 ± 7.8a	18.49 ± 0.76a
	normal		110.3 ± 2.1	3.65 ± 0.03

[a] Statistical significance: a, $p < 0.001$ vs normal rats; b, $p < 0.001$ vs diabetic control rats.

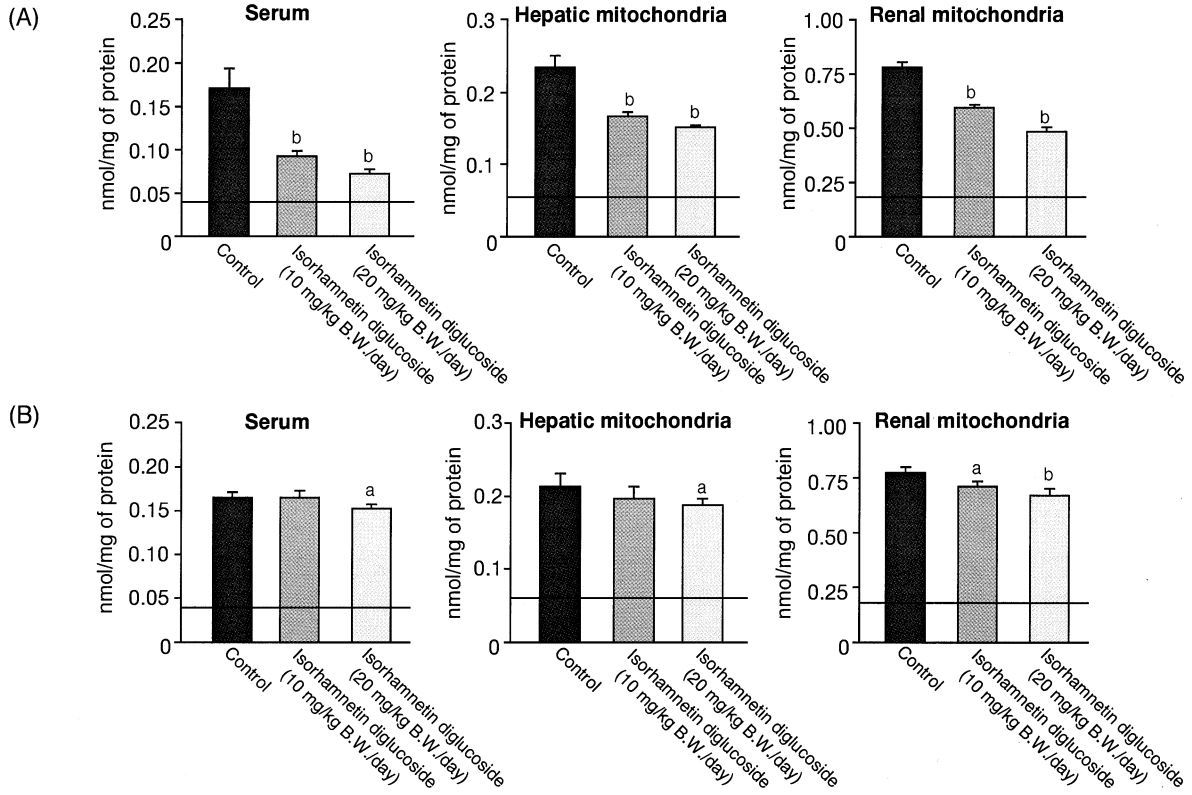

Figure 1. Effect of isorhamnetin diglucoside on TBA-reactive substance levels after oral (A) or intraperitoneal (B) administration. Statistical significance: a, $p < 0.05$; b, $p < 0.001$ vs diabetic control rats. The value of normal rats is expressed as the horizontal line.

Table 2. DPPH Radical Scavenging Activity

compound	IC_{50} (μg/mL)
isorhamnetin diglucoside	179.7 ± 0.8
isorhamnetin	26.7 ± 0.9

2) yielded no chromatographic peaks other than isorhamnetin diglucoside. However, after enzymatic hydrolysis by β-glucosidase, a new peak appeared in the chromatogram, and comparison with the retention time (42 min) of an authentic sample proved it was isorhamnetin, as shown in **Figure 2**. Although there was no isorhamnetin peak and nothing but isorhamnetin diglucoside present before incubation, the conversion of isorhamnetin diglucoside to isorhamnetin by β-glucosidase increased in a time-dependent manner (32% change of isorhamnetin diglucoside after 7 h).

DPPH Radical Scavenging Activities of Isorhamnetin Diglucoside and Isorhamnetin. Table 2 shows the DPPH radical scavenging activity of isorhamnetin diglucoside and isorhamnetin, its aglycone. Isorhamnetin inhibited DPPH formation by 50% at a concentration of 26.7 μg/mL (IC_{50}), whereas isorhamnetin diglucoside, with a high IC_{50} of 179.7 μg/mL, showed weak DPPH radical scavenging activity.

Effects of Isorhamnetin on Glucose, Glycosylated Protein, and TBA-Reactive Substance Levels. Figure 3 shows the effects of isorhamnetin on rats with STZ-induced diabetes. We evaluated the glucose, glycosylated protein, and TBA-reactive substance levels of serum and hepatic and renal mitochondria. Intraperitoneal administration of isorhamnetin at 10 mg/kg of body weight/day reduced the serum levels of glycosylated protein as well as glucose. The level of glucose declined from 557.2 to 481.9 mg/dL and that of glycosylated protein fell from 20.58 to 16.41 nmol/mg of protein. Isorhamnetin also showed protective activity against lipid peroxidation. The TBA-reactive substance levels of serum and hepatic and renal mitochondria decreased > 20% compared with those of control diabetic rats. The TBA-reactive substance levels significantly declined by 23 and 27% in hepatic and renal mitochondria, respectively.

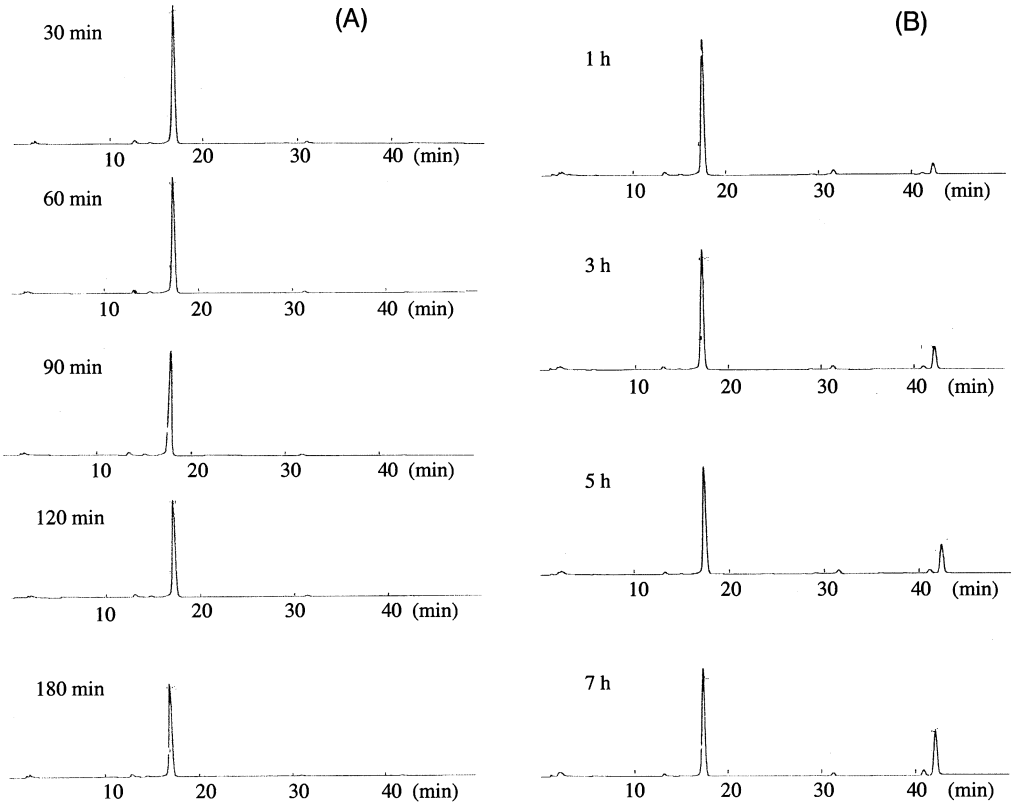

Figure 2. Time course of conversion of isorhamnetin diglucoside by HCl (pH 2) (A) and β-glucosidase (B).

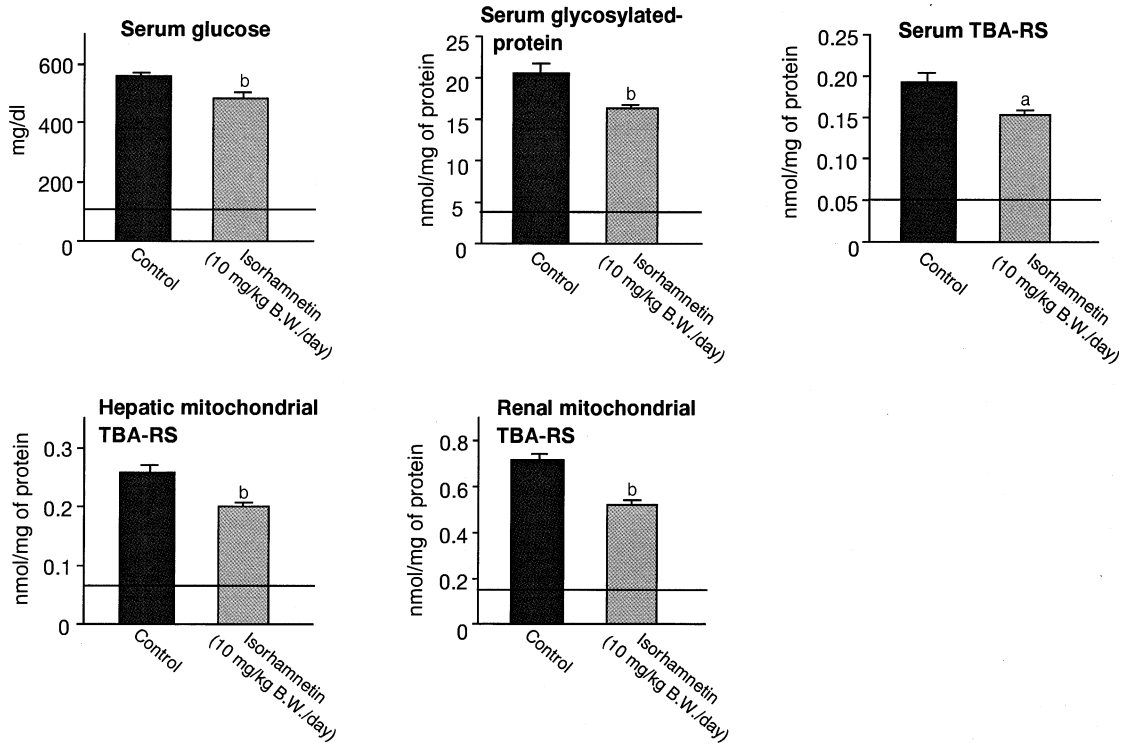

Figure 3. Effect of isorhamnetin on glucose, glycosylated protein, and TBA-reactive substance levels after intraperitoneal administration. Statistical significance: a, $p < 0.01$; b, $p < 0.001$ vs diabetic control rats. The value of normal rats is expressed as the horizontal line.

DISCUSSION

Recently, much attention has been focused on antioxidants in food that are potential compounds for preventing diseases caused by oxidative stress including diabetes because of their distinctive biological activity and low toxicity. In our previous study on mustard leaf, we found that the EtOAc and BuOH fractions had stronger DPPH and hydroxyl radical scavenging activities than the CH_2Cl_2 and H_2O fractions (*20*). In view of these results and the yields by weight from dried mustard leaf (EtOAc fraction, 0.07%; BuOH fraction, 0.85%), we carried out in vivo and in vitro experiments with the main compound

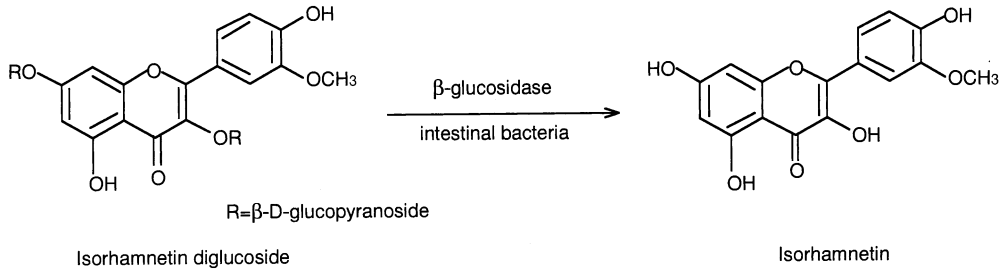

Figure 4. Transformation of isorhamnetin diglucoside to isorhamnetin in vivo.

of the BuOH fraction, isorhamnetin diglucoside (~1.5% of this fraction), using rats with STZ-induced diabetes.

Diabetes can be produced in animals by administering STZ, which is toxic to β-cells and is widely used for the induction of experimental diabetes mellitus, resulting in the production of active oxygen species (*21*). Scavengers of oxygen radicals are effective in preventing diabetes in these animal models (*2*, *22*). Therefore, we employed such a diabetic animal model system to examine the antioxidant effects of isorhamnetin diglucoside isolated from mustard leaf on oxidative stress.

Hyperglycemia and glycation of proteins are associated with the development of diabetic complications, resulting in the generation of oxygen free radicals (*23*). In particular, increased glycation of collagen and plasma proteins in subjects with diabetes may stimulate the oxidation of lipids, which may in turn stimulate autoxidative reactions of sugars, enhancing damage to both lipids and proteins in the circulation and the vascular wall, continuing and reinforcing the cycle of oxidative stress and damage (*2*). Bunn et al. (*24*) reported that 5-HMF is involved in the nonenzymatic browning process, and nonenzymatically bound glucose in serum was found to be released as 5-HMF (*25*). Therefore, we evaluated 5-HMF levels to determine the extent of glycosylation of serum proteins. The results on glucose and glycosylated protein levels indicate that oral administration of isorhamnetin diglucoside might prevent the pathogenesis of diabetic complications caused by impaired glucose metabolism and glycosylation of serum proteins, eventually resulting in improvement of the diabetic pathological conditions.

Oxidative stress is associated with the peroxidation of cellular lipids, which is determined by measuring TBA-reactive substance levels. The concentration of lipid peroxidation products may also reflect the oxidative stress associated with the diabetic condition. Baynes (*2*) and Kakkar et al. (*26*) reported that tissue and blood malondialdehyde levels of rats with STZ-induced diabetes increased due to lipid peroxidation. On the basis of these results, we evaluated TBA-reactive substance levels to determine the degree of oxidative damage in diabetic rats and found out that it was significantly higher in the diabetic control group compared with the normal group. This result suggests that the increased TBA-reactive substance levels in diabetic rats result in increased levels of oxygen free radicals, which attack the polyunsaturated fatty acids in cell membranes and cause lipid peroxidation, leading to pathological conditions. In contrast, orally administered isorhamnetin diglucoside reduced the TBA-reactive substance levels, indicating that isorhamnetin diglucoside may attract a lot of attention as a new therapeutic agent for diabetes in view of its inhibition of the lipid peroxidation and free radical production caused by STZ.

The effects of orally and intraperitoneally administered isorhamnetin diglucoside on glucose metabolism and lipid peroxidation differed. Orally administered isorhamnetin diglucoside resulted in the improvement of glucose metabolism and protection against lipid peroxidation, whereas after intraperitoneal administration, this compound had little effect, if any. Therefore, we hypothesized that after oral administration, isorhamnetin diglucoside is converted to an active metabolite in the stomach or intestine and this metabolite has antioxidant properties. There is a similar report of the antioxidant mechanism and the function of its metabolites in vivo (*27*). Thus, it is important to determine how antioxidants in food are metabolized in vivo and how antioxidant metabolites function in vivo. We also found differences between in vivo and in vitro results with isorhamnetin diglucoside. It had little DPPH radical scavenging activity in vitro, whereas after oral administration, it had a strong antioxidative effect in a diabetic animal model in vivo. Hence, to investigate the mechanism responsible for the function of isorhamnetin diglucoside in vivo after oral administration, we attempted to hydrolyze isorhamnetin diglucoside with HCl at pH 2 (reflecting conditions in the stomach) or β-glucosidase and compared the antioxidant activity of isorhamnetin diglucoside with that of its metabolite. From the results, we assume that isorhamnetin diglucoside, a main compound of mustard leaf, is metabolized by intestinal bacteria to isorhamnetin, which plays a central antioxidant role in vivo (**Figure 4**).

Isorhamnetin diglucoside is a kind of flavonoid present in vegetables and fruits. Flavonoids generally occur in foods as *O*-glycosides with bound sugars, usually at the C3 position. Until recently, it has been assumed that the flavonoid glycosides cannot be easily absorbed from the small intestine and the cleavage of β-glycoside linkage will not occur until the compounds reach the microflora in the large intestine (*28*). However, the β-glucosidase capable of efficiently hydrolyzing various naturally occurring flavonoid glycoside is found abundantly in the small intestine as well as the large intestine of humans (*29*−*31*). Although the rate and extent of deglycosylation depend on the structure of the flavonoid and the position/nature of the sugar substitutions, the flavonoids with glucose at the 3- and 7-positions are all substrates for the β-glucosidase (*31*). On the basis of these studies, isorhamnetin diglucoside from mustard leaf is considered to be hydrolyzed efficiently to its aglycone, isorhamnetin.

The deglycosylation of flavonoids by human β-glucosidase could be an important first step in their uptake metabolism, excretion, and biological activity. In addition, biological activity depends on the presence or absence of the glycoside residue (*32*). The aglycone is likely to have a greater biological effect than the glycoside, so deglycosylation via a β-glucosidase activity would be an important step in metabolism. Cyanidine rather than cyanidine glucosides acts as an antioxidant in living systems and, after ingestion, the latter can be hydrolyzed by β-glucosidase of intestinal bacteria (*33*). In addition, Miyake et al. (*22*) reported that the antioxidant activities of the aglycones of the flavonoid glycosides in lemon fruit were stronger than those of the flavonoid glycosides themselves. We could also

confirm that isorhamnetin diglucoside is metabolized by intestinal bacteria to isorhamnetin. Isorhamnetin exhibited antioxidant activities in rats with STZ-induced diabetes in vivo as well as in vitro, reducing the levels of DPPH radical, serum glucose, and glycosylated proteins and protecting serum and tissue mitochondria against lipid peroxidation. From these results, we conclude that the antioxidant activity of the active compound from mustard leaf is attributable to isorhamnetin not to isorhamnetin diglucoside itself. In view of the antioxidant activities observed in vivo as well as in vitro, mustard leaf containing high levels of isorhamnetin diglucoside seems to be effective for reducing oxidative stress and is expected to contribute to the prevention of life style-related diseases.

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