Protective effects of dietary kaempferol glycoside components from unripe soybean (Edamame, *Glycine max* L. Merrill. ‘Jindai’) leaves and their serous metabolite on carbon tetrachloride-induced liver injury mice

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Abstract The study investigated the protective effects of kaempferol galactoside (KG) components in mice, which were separated from Jindai soybean leaves (JDL) and mainly composed by two kaempferol galactosides. Further, KG-related metabolites in serum of mice were identified by ToF-MS. Results showed that both JDL and KG prevented the CCl₄-induced increases in serum aspartate aminotransferase and serum alanine aminotransferase. Additionally, mice treated with KG had significantly decreased TBARS and TNF- α levels, compared to CCl₄-treated mice. Serous analysis showed that kaempferol, glucuronidated kaempferol and methylated kaempferol with a glucuronic acid moiety were identified in the serum of mice fed unripe soybean leaves or kaempferol galactosides isolated from the leaves. The results indicated that kaempferol 3-*O*-galactoside connected to other glycosides via galactose might be hydrolyzed in the gastro-intestinal tract and/or epithelium cells to release kaempferol, followed by glucuronidation and/or methylation in the liver to contribute to a reduction in liver injury. The use of raw leaves

containing kaempferol galactosides as food materials may contribute to a reduction in oxidation-related diseases.

Keywords Kaempferol glycoside · Liver injury · Mice · Metabolite

Introduction

Unripe soybean, Edamame, is prepared by treating the pod with boiling water over a short period. Its consumption is increasing in Japan, particularly when eaten in combination, it enhances the taste of beer. Leaves of unripe soybeans, which are green in color, are considered to contain high amounts of nutrients and polyphenols yet are often discarded without being used. Jindai soybean (*Glycine max* L. Merrill. ‘Jindai’) is a local variety, cultivated in Shinjyo, Yamagata Prefecture, Japan and is characterized by its larger leaves, higher stems and larger unripe beans, in comparison with other varieties. In addition, Jindai soybeans from the Shinjyo area are almost all cultivated without the use of agricultural chemicals, suggesting that its leaves could be used as raw food material and used to extract compounds with health promoting activities. We previously reported that a kaempferol glycosides-rich fraction, containing kaempferol 3-*O*- β -D-glucopyranosyl (1 $\rightarrow$ 2)-*O*-[α -L-rhamnopyranosyl (1 $\rightarrow$ 6)]- β -D-galactopyranoside, kaempferol 3-*O*- β -D-glucopyranosyl (1 $\rightarrow$ 2)-*O*-[α -L-rhamnopyranosyl (1 $\rightarrow$ 6)]- β -D-glucopyranoside, kaempferol 3-*O*- β -D-(2-*O*- β -D-glucopyranosyl) galactopyranoside and kaempferol 3-*O*- β -D-(2,6-di-*O*- α -L-rhamnopyranosyl) galactopyranoside, had anti-diabetic effects in type 2 diabetes model mice, KK-A^y (Zang et al. 2011). The result also indicated that these compounds, and leaves containing these compounds, may have other

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physiological functions, such as protection from oxidative stress, since it is known that the progression of diabetes is closely related to an increase in oxidation in vivo (Kamalakannan et al. 2003; Nagasawa et al. 2003; Nicolle et al. 2011). Previous studies have shown that kaempferol glycoside-rich fractions prepared from the aerial parts of Egyptian *Vicia calcarata* Desf. (Singab et al. 2005), young seedpods of garden peas (Murakami et al. 2001) and boysenberry leaves (Kubomura et al. 2006) have protective effects against liver injury in mice, and we suggest that kaempferol glycosides contained in unripe soybean leaves may also be effective in protecting against liver injury. To our acknowledgement, the relationship between the sugar moiety and/or the amounts connected to the 3-position of kaempferol and protective activity against liver injury has not been examined. In this study, the hepato-protective effects of kaempferol 3-*O*-glycosides, against liver injury were examined in relation to its absorption from the gastrointestinal tract and metabolism. We also studied the hepato-protective effect of kaempferol glycosides in the raw leaves which would be invaluable in the production of foods for health-promoting effects.

Materials and methods

Preparation of kaempferol glycosides (KG)

Jindai soybean (*Glycine max.* L. Merrill. 'Jindai') leaves were collected in September 2008 from a farm in the Mogami area of Yamagata Prefecture in Japan. The leaves were washed with water and some were freeze-dried until use in animal experiments (JDL, Jindai soybean leaves). The remaining leaves were dried in the shady open air, and extracted with 70% methanol (MeOH) at 60–65 °C under reflux for 3 h. The evaporated extract was dissolved in MeOH, before adding CHCl₃ and H₂O to give a mixture of CHCl₃/MeOH/H₂O (1:1:2). After concentrating the upper phase of the mixture, it was applied to an ion-exchange resin column (2.5 cm i.d. × 20 cm; Diaion, HP-20; Mitsubishi Co., Japan). After washing the column with H₂O, the phenolic compounds were successively eluted with aqueous 20%, 50% and 100% MeOH solutions (500 ml). The eluate from the aqueous 20% MeOH solution was evaporated and dissolved in 100% MeOH, and then applied to silica gel column chromatography (prepared with *n*-hexane), using *n*-hexane/EtOAc, followed by EtOAc/MeOH as the developing solvents. The fraction containing kaempferol 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl) galactopyranoside (compound 1 in Fig. 1) and kaempferol 3-*O*-β-D-(2,6-di-*O*-α-L-rhamnopyranosyl) galactopyranoside (compound 2 in Fig. 1) was collected by preparative HPLC, and then evaporated for use in the animal experiments as the

kaempferol glycosides fraction (KG). The chemical structures of these compounds were confirmed by comparison of their retention times on the HPLC chromatogram with authentic samples previously prepared from the leaves of unripe soybeans (Zang et al. 2011). Preparative HPLC was performed using a Develosil C30-UG5 column (20 mm i.d. × 250 mm; Nomura Chemical Co., Aichi, Japan) with solvent systems composed of 5% CH₃CN (MeCN) in 1% acetic acid (solvent A) and 40% MeCN (solvent B). A linear gradient of 0–100% of solvent B in solvent A over the course of 180 min at a flow rate of 2 mL/min was used. Analytical HPLC for identification of flavonoids in KG was performed as described above for the preparative HPLC, except that a Develosil C30-UG5 column (4.6 mm i.d. × 250 mm; Nomura Chemical Co., Aichi, Japan) was used and the flow rate was 0.8 mL/min. The amounts of compound 1 and 2 in the freeze-dried leaves of Jindai unripe soybean (JDL) was about 6 and 10 mg/g dry weight, respectively, when measured by HPLC using isolated compounds 1 and 2 as authentic samples (Zang et al. 2011).

Animal experiment

Male ddY mice, 7–8 weeks old, with an average weight of 34 g were purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and cared for according to the institutional guidelines of Yamagata University. Mice were housed individually under a 12:12 h light–dark cycle at 22 ± 2 °C and 40–60% humidity. After acclimatizing for 5 d, the mice were randomly divided into 5 groups of 7 mice and fed either a basal diet (CON and CON* groups), basal diet with freeze-dried leaves (5%) from unripe Jindai soybeans (JDL* group), basal diet with 0.2% kaempferol glycosides fraction (KG* group) or the basal diet with 0.1% kaempferol (K* group). The composition of each experimental diet is shown in Table 1. The diets and water were given for 5 d ad libitum. Body weight and dietary intake were measured daily. All mice were deprived of their respective diet for 8 h before intraperitoneal injection of a mixture containing 30 μL of carbon tetrachloride and 30 μL of olive oil (CON*, JDL*, KG* and K* groups) or 30 μL of olive oil (CON group). Mice were anesthetized with Nembutal (Dainippon Pharmaceutical Co., Osaka, Japan) 22 h after the intraperitoneal injection and blood was collected by cardiac puncture, followed by centrifugation at 3000×g for 20 min to obtain serum (Figs. 2, 3).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum were measured according to a method described previously (Igarashi et al. 2008). One unit of enzyme activity was defined as the amount of enzyme to transform 1 μmol substrate per min per liter of serum (μmol/min/L of serum at 25 °C).

Fig. 1 HPLC chromatogram of the kaempferol glycosides fraction (KG) prepared from unripe soybean leaves (*Glycine max* L. Merrill. ‘Jindai’). HPLC conditions: column, Develosil C-30-UG-5 (4.6 i.d. × 250 mm, Nomura Chemical Co., Aichi, Japan); development,

a linear gradient of 0–100% of solvent B (40% MeCN) in solvent A (5% MeCN in 1% acetic acid) over the course of 180 min at a flow rate of 0.8 mL/min; detection at 330 nm

Table 1 Composition of the experimental diets (%)

	CON	CON*	JDL*	KG*	K*
Casein	20	20	20	20	20
α -Cornstarch:Sucrose = 2:1	62.95	62.95			62.85
Cellulose powder	5	5	5	5	5
Corn oil	7	7	7	7	7
Mineral mixture	3.5	3.5	3.5	3.5	3.5
Vitamin mixture	1	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Jindai soybean leaves (JDL)			5		
Kaempferol glycoside fraction (KG)				0.2	
Kaempferol (K)					0.1
Total (%)	100	100	100	100	100

*Groups injected with carbon tetrachloride after feeding the respective diet

To determine the metabolites of kaempferol glycoside (KG) and kaempferol, 0.2 mL serum was hydrolyzed with sulfatase containing glucuronidase (Sigma Co., Ltd.) at pH 6.0 for 30 min. The solution was applied to a Sep-Pak C18 Cartridge (5 g, Waters Co., Ltd.) and washed with distilled water. MeOH was used to elute from the Sep-Pak and the eluate was dispersed into distilled water and chloroform at a ratio of 1:2:1 (MeOH:H₂O:CHCl₃, v/v/v). The obtained upper phase was evaporated and dissolved in MeOH. The MeOH soluble compounds were analyzed by high-resolution electrospray ionization (HRESI–ToF–MS) with UPLC (Xevo QToF MS, Waters,

Milford, MA, USA) in the negative mode, using a BEH C-18 column (0.5 × 50 mm, 1.7 μ m; Waters, USA). The solvent system was composed of 0.1% HCOOH in water (solvent A) and 0.1% HCOOH in MeCN (solvent B). Developing solvent was eluted at a flow rate of 0.3 mL/min with gradient elution (0 min: 90% A, 6 min: 60% A, 7 min: 60%, 8 min: 10% A, 9–13 min: 90% A). Cone and desolvation gas flows were set at 50 and 800 L/h, respectively. The capillary voltage was set at 2.5 kV, the source temperature at 120 °C, and the desolvation temperature at 400 °C.

Fig. 2 Serum ALT and AST activities in groups treated with an intraperitoneal injection of carbon tetrachloride (CON*, JDL*, KG* and K* groups), or non-treated (CON group) after feeding the respective diet. CON and CON*, control diet fed-group; JDL*, unripe Jindai soybean leaves-fed-group; KG*, kaempferol glycosides-fed group; K*, kaempferol-fed group. Values not sharing a common letter are significantly different at $p < 0.05$

Statistical analysis

Each value in the animal experiment is given as the mean $\pm$ standard error. Differences between groups were determined by Berformin's method, following a one-way analysis of variance (ANOVA). Mean values were significantly different when $p < 0.05$.

Results and discussion

The mice fed with kaempferol glycoside (KG), kaempferol (K) or Jindai soybean leaves showed lower ALT and AST activities, compared with mice fed the control diet, suggesting that part of the protective effect of JDL against liver injury may be caused by kaempferol or its glycosides, kaempferol 3-*O*- β -D-(2-*O*- β -D-glucopyranosy) galactopyranoside or kaempferol 3-*O*- β -D-(2,6-di-*O*- α -L-rhamnopyranosyl) galactopyranoside contained in the JDL. JDL also contains isoflavones and their contribution to mitigation of liver injury needs to be studied (Khan and Sutana 2011). The hepato-protective effects of kaempferol with galactose

Fig. 3 Serum TBARS and TNF- α in groups treated with an intraperitoneal injection of carbon tetrachloride (CON*, JDL*, KG* and K* groups), or non-treated (CON group) after feeding the respective diet. CON and CON*, control diet fed-group; JDL*, unripe Jindai soybean leaves-fed-group; KG*, kaempferol glycosides-fed group; K*, kaempferol-fed group. Values not sharing a common letter are significantly different at $p < 0.05$

at the 3-position has not been reported, this may be due to their low content in plants. There were no significant differences in both the ALT and AST activities between the KG* and K* groups, suggesting that the effect of KG might be exerted by its kaempferol moiety. The presence of kaempferol, methylated kaempferol, kaempferol with a glucuronic acid moiety, and methylated kaempferol with a glucuronic acid moiety in serum treated with sulfatase containing glucuronidase (Fig. 4) indicates that kaempferol glycosides might be absorbed directly and/or after hydrolysis in the gastro-intestinal tract and/or epithelium cell and then methylated and/or glucuronidated in the liver. A small amount of methylated kaempferol, kaempferol with a glucuronic acid moiety, and methylated kaempferol with a glucuronic acid moiety was present in serum treated with sulfatase containing glucuronidase and this may be due to incomplete hydrolysis.

In Fig. 4a, b, c, d, it was considered that total ion chromatogram (bottom section), chromatograms of ions detected at m/z 285.03 ($\pm$ 0.05) (middle section) and at m/z 299.05 ($\pm$ 0.05) (top section) were due to molecular weight of kaempferol, and its methyl ether.

Fig. 4 UPLC/MS chromatograms of the serum of mice fed the control diet (a), or kaempferol glycosides (b), kaempferol (c) and freeze-dried young soybean leaves (d) added diets for 4 days before intraperitoneal injection of carbon tetrachloride. Serum was treated with sulfatase containing glucuronidase for 30 min, and then treated as described in the text. The precise mass of compounds a, e and g

was calculated as 299.0556 ($C_{16}H_{11}O_6$, kaempferol methyl ester). The precise mass of compound b and c were calculated as 483.0564 ($C_{21}H_{16}O_{12}Na$, kaempferol moiety + glucuronic acid moiety + Na) and 475.0877 ($C_{22}H_{19}O_{12}$, kaempferol moiety + glucuronic acid moiety + CH_3). The precise mass of compounds d, f, and h were calculated as 285.0399 ($C_{15}H_9O_6$, kaempferol)

As shown in the ion chromatograms in the middle and top sections of Fig. 4a, peaks with precise molecular weights, which were consistent with those of kaempferol ($[M-H]$: 285.0399, calcd. for $C_{15}H_9O_6$) and of its methyl ether ($[M-H]$: 299.0556, calcd. for $C_{16}H_{11}O_6$), described and discussed later, were not found in the serum of mice fed the control diet. This result showed that kaempferol and its methyl ether were not present in the serum of mice fed with the control diet.

On the other hand, Pseudo molecular ions $[M-H]$ at m/z 299.0563 (compound a in the top section of Fig. 4b: $R_t = 5.04$, 299.0556, calcd. $C_{16}H_{11}O_6$) in mice fed kaempferol glycoside, and at m/z 299.0563 (compound c in the top section of Fig. 4c: $R_t = 5.04$, 299.0556, calcd. for $C_{16}H_{11}O_6$) in mice fed kaempferol, and moreover at m/z 299.0576 (compound g in the top section of Fig. 4d: $R_t = 5.04$, 299.0556, calcd. for $C_{16}H_{11}O_6$) in mice fed Jindai-

soybean leaves, along with ions considered as their respective fragment at m/z 285.0407 (compound d in the middle section of Fig. 4b, 285.0399, calcd. for $C_{15}H_9O_6$, kaempferol moiety), at m/z 285.0390 (compound f in the middle section of Fig. 4c, 285.0399, calcd. for $C_{15}H_9O_6$, kaempferol moiety) and at m/z 285.0404 (compound h in the middle section of Fig. 4d, 285.0399, calcd. for $C_{15}H_9O_6$, kaempferol moiety) showed good agreement with those of methylated kaempferol and kaempferol, respectively. These results indicated that kaempferol glycosides contained in Jindai-soybean leaves might be first hydrolyzed to liberate kaempferol in the gastro-intestinal tract, next methylated in the intestinal epithelium cells and/or in the liver, as reported in rats fed with kaempferol derivatives (Chen et al. 2014),

Pseudo molecular ion ($[M-H+Na]$) with m/z 483.0562 (483.0539, calcd. for $C_{21}H_{16}O_{12}Na$, kaempferol

moiety + glucuronic acid moiety + Na) at $R_t = 2.86$ in Fig. 4b (compound b in the middle section), which was found along with its Na-free type ($[M-H]$) with m/z 461.0735 (461.0720, calcd. for $C_{21}H_{17}O_{12}$, kaempferol moiety + glucuronic acid moiety) in the MS chromatogram of compound b with $R_t = 2.86$ (Fig. 5a), indicated that kaempferol could be glucuronidated in vivo, similar to quercetin glycosides (Guo et al. 2010; Qiao et al. 2011). Pseudo molecular ion ($[M-H]$) with m/z 475.0870 (475.0877, calcd. for $C_{22}H_{19}O_{12}$, kaempferol moiety + glucuronic acid moiety + CH_3) at $R_t = 3.52$ in the middle section of Fig. 4b (compound c), and that found in MS chromatogram of compound c with $R_t = 3.52$ (Fig. 5b) and moreover, its fragment ions at m/z 285.0406 (285.0399, calcd. for $C_{15}H_9O_6$, kaempferol moiety) indicated that kaempferol liberated from kaempferol glycoside in the gastro-intestinal tract also could be glucuronidated

before or after its methylation in the epithelium cells and/or liver, similar to quercetin glycosides (Guo et al. 2010; Qiao et al. 2011). These results indicated that kaempferol, which might be liberated from its glycosides, compound 1 (kaempferol 3- O - β -D-(2- O - β -D-glucopyranosyl) galactopyranoside) and compound 2 (kaempferol 3- O - β -D-(2,6-di- O - α -L-rhamnopyranosyl) galactopyranoside), could be absorbed from the gastro-intestinal tract, probably after hydrolysis to their aglycone (kaempferol), then contribute to CCl_4 -induced liver injury by scavenging radicals which were produced by the action of CCl_4 (Jin et al. 2005).

It was reported that quercetin 3-sophorotrioside and kaempferol 3- O -sophorotrioside contained in young seed-pods of garden peas (*Pisum sativum* L.) reduced galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes, and that quercetin 3- O -sophorotrioside was effective in suppressing CCl_4 -induced liver injury in mice

Fig. 5 MS spectra of compounds with retention times at 2.86 (compound b) and 3.52 min (compound c) on MS chromatogram at m/z 285.03 in Fig. 4b (middle section)

(Nagasawa et al. 2003). It was also reported that a mixture of kaempferol-3-*O*- β -D-glucopyranoside (astragalin), quercetin-3-*O*- β -D-glucopyranoside (isoquercitrin), rutin, kaempferol-3, 5-di-*O*- β -D-glucopyranoside, and quercetin-3, 5-di-*O*- β -D-glucopyranoside can ameliorate CCl₄-induced liver injury in rats (Murakami et al. 2001). Hepatoprotective effect of kaempferol 3-*O*- β -D-(2-*O*- β -D-glucopyranosyl) galactopyranoside and kaempferol-3-*O*- β -D-(2,6-di-*O*- α -L-rhamnopyranosyl) galactopyranoside (mauritinin), which have a galactose moiety at the 3-position that is connected to other sugars, has not yet been reported. The presence of kaempferol galactoside is reported in *Crassocephalum crepidioides* from the Okinawa Islands in Japan (Aniya et al. 2005). It was reported that kaempferol with galactoside at the 3-position and connected with other sugar moieties via the galactose moiety, can be hydrolyzed in the gastro-intestinal tract and absorbed the same as kaempferol with a glucose moiety at the 3-position (Banaszewski et al. 2013). The report showed that isorhamnetin 3-*O*-galactoside had protective activity against CCl₄-induced liver injury and this suggests that both isorhamnetin 3-*O*-galactoside and kaempferol 3-*O*-galactoside, with other sugars linked via the galactose, could be hydrolyzed to liberate kaempferol in the gastro-intestinal tract (Kim et al. 2012).

The compounds used in this experiment are present in *Lysimachia mauritiana* and *Galega officinalis* (Champavier et al. 2000; Yasukawa and Takido 1987) and a hepatoprotective effects may be expected in these plants, in addition to other foodstuffs.

At present, almost all unripe soybean leaves containing kaempferol glycosides, as well as isoflavones, are discarded without use. Their use as raw materials in the production of teas and green leaf juice may be invaluable in contributing to protection against diseases such as liver injury, especially if the soybeans could be grown without agricultural chemicals, or if the chemicals could be removed before use.

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