Unique Uptake and Transport of Isoflavone Aglycones by Human Intestinal Caco-2 Cells: Comparison of Isoflavonoids and Flavonoids

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ABSTRACT Soy isoflavonoids have attracted much attention because of their estrogenic activity. To study the intestinal absorption of the isoflavonoids, we investigated the cellular uptake and metabolism of genistein and daidzein and their glucosides, genistin and daidzin, by Caco-2 cell monolayers as a model of the human intestinal epithelium. When Caco-2 monolayers were incubated with genistein or daidzein at 10 μmol/L, from the apical (mucosal) side, aglycone was lost from the apical solution for 2.0 h (P < 0.05) and the glucuronide/sulfates appeared at the level of 1–2 μmol/L. In the basolateral (serosal) solution, both intact aglycones and their glucuronide/sulfates increased (P < 0.05) with time and reached ~20 and 15% of the initial dose, respectively. The transport of their glucosides, genistin and daidzin, through Caco-2 monolayers was less than one tenth that of the aglycones. The cellular metabolism of genistein was compared with quercetin, kaempferol, luteolin and apigenin. Only genistein aglycone was transported intact to the basolateral solution. Transport was greater (P < 0.05) than that of flavonoid aglycones and was without an appreciable decrease of transepithelial resistance. Radical scavenging activity was not related to the susceptibility to conjugation of flavonoids/isoﬂavonoids. Affinity to the liposomal membrane was increased in the order of genistin = daidzin < genistein < flavonoid aglycones. These results strongly suggest that isoflavone aglycones are taken up into enterocytes more efficiently than their glucosides because of their moderate lipophilicity. Furthermore, they are generally transported to the basolateral side in intact form in contrast to flavonoids, probably due to their unique isoﬂavonoid structure.


KEY WORDS: • isoflavones • aglycone • glucoside • intracellular metabolism • Caco-2 cells

Isoflavonoids, which possess a diphenylpropane structure in which the B-ring is located at the 3-position, are major dietary components of soybeans and soy products. The soy isoflavones, genistein and daidzein, and their 7-glucosides (genistin and daidzin) (Fig. 1) have recently received much attention because of their potential benefits as phytoestrogens in decreasing the risk of a variety of diseases. There are several reviews on soy (and isoflavonoid) intake and health promotion, including the prevention of cancers (1,2), osteoporosis (3) and cardiovascular diseases (4). It is essential to evaluate the bioavailability of isoflavones to estimate the benefit of soybean and soy product intakes. We reported earlier that the isoflavone aglycones, which are present in fermented soy foods (5), are absorbed more easily than their glucosides in humans (6). Andlauer et al. studied the absorption and metabolism of genistein (7) and genistin (8) using isolated rat small intestine. These reports also suggested that more genistin than genistein was absorbed in rat small intestine. However, the reason why isoflavone aglycones were absorbed more efficiently than their glucosides is not known.

The Caco-2 cell line, which is derived from human colon adenocarcinoma (9), exhibits enterocyte-like characteristics and has been used widely as an in vitro model of absorption by intestinal epithelial cells (10–13). Using Caco-2 cell monolayers, we previously examined the cellular uptake and metabolism of quercetin, a typical flavonoid in plant foods. We found that quercetin aglycone was taken up into Caco-2 cells and metabolized to its conjugated forms (glucuronide or sulfate) more efficiently than its glucosides, and reported that lipophilicity contributed to the efficient uptake of aglycone into Caco-2 cells (14). In this study, we investigated the cellular uptake and metabolism of genistein and daidzein, and their glucosides by Caco-2 cells to clarify the process of isoflavonoid absorption in the small intestine. In addition, isoflavonoid metabolism by Caco-2 cells was compared with that of quercetin and other flavonoids that possess diphenylpropane structures with a B-ring at the 2-position.

MATERIALS AND METHODS

Materials. Genistein and daidzein were synthesized according to the method of Wåhåll and Hase (15). Genistin and daidzin were...
corning costar, NY) in 24-well plates at a density of 2.5 cells/insert (0.33 cm²/insert). The basolateral (serosal) and apical (DMSO³) and applied to HPLC. The mobile phase at a concentration of iso flavonoids/ flavonoids was adjusted to 10 µmol/L. These test solutions were added on the apical side of Caco-2 monolayers and incubated for 0.5, 1.0 or 2.0 h at 37°C. After the incubation, the apical and the basolateral solutions were collected. Cellular extracts were prepared by incubating the whole inserts with methanol for 30 min. The apical and the basolateral solutions and the cellular extracts were each divided into two aliquots and evaporated using a centrifugal vaporizer. Half of either apical solution, basolateral solution or cellular extracts was mixed with 20U of a sulfatase type H-5 solution in 100 mmol/L acetate buffer (0.1 mL, pH 5.0) and incubated at 37°C for 45 min. Then, the same volume of methanol was added to the mixture and centrifuged at 10,000 × g for 10 min. The resulting supernatant solution was used as a sulfatase-treated sample. The other half was dissolved and used as an untreated sample. The amounts of the metabolites (glucuronide/sulfates) were calculated by the difference between the amounts of iso flavonoids/flavonoids from sulfatase-treated samples and those from untreated samples, as described previously (16). Because sulfatase type H-5 possesses sulfatase, glucuronidase and glycosidase activities, the amounts of the glucuronide/sulfates from the samples of the glucosides (genistin and daidzin) were calculated by subtracting the amounts of aglycone and glucosides obtained in untreated samples from that of total isoflavone in sulfatase-treated samples. Other metabolized forms, such as methylated forms, were not identified in this study.

All iso flavonoids/flavonoids were identified by HPLC analysis using a C18 column (TSK gel ODS-80Ts, Tosoh, Tokyo, Japan). The mobile phase at a flow rate of 1.0 mL/min was composed of methanol/water/acetic acid (36:62:2, v/v/v) for genistein, daidzein and genistin, and methanol/water/acetic acid (32:60:2, v/v/v) for daidzin. The eluate was monitored with a UV detector at 256 nm. For the analyses of quercetin, kaempferol, luteolin and apigenin, a mobile phase of methanol/water/acetic acid (46:52:2, v/v/v) was used. The eluate was monitored with a UV detector at 365 nm. The concentration of their aglycones was calculated using internal standards, kaempferol or quercetin, in the respective experiments. Under these conditions, the analytical limit was 0.2 µmol/L.

Measurement of the transepithelial resistance of Caco-2 monolayers. The transepithelial electrical resistance (TEER) value of the Caco-2 monolayers was measured according to the methods of Hidalgo et al. (10) with a Millicell-ERS instrument (Millipore, Bedford, MA). The measurements were performed before and after 10 min and 2 h of incubation with isoflavonoids/flavonoids.

Estimation of the affinity to the liposomal membrane. The affinity of isoflavonoids and flavonoids to large unilamellar vesicles (LUV) was determined by a method described previously (17). Briefly, chloroform solutions of purified egg yolk PC (18) and cholesterol were mixed in a test tube. The solvent was removed with a stream of nitrogen followed by evaporation under vacuum. The residue was dispersed in Tris-HCl buffer (10 mmol/L, pH 7.4) with 0.5 mmol/L diethylenetriaminepentaacetic acid. The suspension was mixed vigorously followed by ultrasonic irradiation. LUV were obtained by the extrusion method using a LiposoFast apparatus (Avestin, Ottawa, Canada). The preparation was made just before the experiments. Each isoflavonoid/flavonoid was added to the LUV suspension (final concentration of flavonoids = 20 µmol/L). After the mixture was incubated in the dark at 37°C for 10 min, it was filtered through an ultrafiltration membrane (UFC3TG002, Milli-pore, pore size = 10 nm) by centrifugation at 10,000 × g for 40 min. The filtrated and unfiltered solutions were recovered. The filter was washed in a mixture of chloroform and methanol (95:5, v/v) and the washed solution added to the unfiltered solution. Both solutions were diluted with methanol and the amounts of isoflavonoids/flavonoids measured by HPLC.

Determination of DPPH radical scavenging activity. Free radical scavenging activity of the aglycones of isoflavonoids and flavonoids was assayed using a stable free radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), according to the method of Blois (19). An ethanol
solution (100 μL) of each compound at different concentrations (10–350 μmol/L) was added to the mixture of 100 mmol/L Tris-HCl buffer (0.9 mL, pH 7.4) and 0.5 mmol/L DPPH (1.0 mL) in ethanol. The solution was mixed vigorously and kept for 20 min in the dark. The free radical scavenging activity of each flavonoid was quantified by the decolorization of DPPH at 517 nm.

**Statistics.** Data are presented as means ± SD. Statistical analyses were carried out with StatView J-4.5 for Macintosh (Abacus Concepts, Berkeley, CA). The significance of differences among different isoflavonoid/flavonoids was tested using one-way ANOVA. Two-way ANOVA or the nonparametric Kruskal-Wallis test was adopted to assess any differences among the incubation time points and among initial compound and metabolites simultaneously. Differences with P < 0.05 were considered to be significant in all experiments and Fisher’s Protected Least Significant Difference test was conducted when appropriate.

**RESULTS**

**Changes of genistein, daidzein and their glucosides in the apical solution.** When isoflavone aglycones, genistein and daidzein (Fig. 1), were applied to the apical side of Caco-2 monolayers at 10 μmol/L, they decreased significantly in the apical solution with incubation time (Fig. 2A). Glucuronyl and sulfated forms of isoflavones appeared at 1.0 h and their contents increased significantly. After 2.0 h of incubation, daidzein glucuronide/sulfates in the apical solution (Fig. 2B) increased significantly more than genistein glucuronide/sulfates (Fig. 2A) (1.72 ± 1.09 μmol/L and 0.70 ± 0.75 μmol/L, respectively, P < 0.05). On the other hand, no changes were observed in the concentrations of genistin (Fig. 2C) and daidzin (Fig. 2D) throughout the incubation period. The aglycones of genistin and daidzin also appeared, although they comprised < 3% of the glucosides. Thus, isoflavonoid aglycones were taken up more easily and to a greater extent by the cell monolayers than their glucosides from the apical side. Caco-2 cells converted isoflavone aglycones to their glucuronide/sulfates and to some extent, returned them to the apical solution.

**Distribution of genistein and daidzin, and their glucosides in cellular extracts after addition to the apical side.** Both of the aglycones and their glucuronide/sulfates were detected in the cellular extracts after incubation of genistein and daidzin with Caco-2 cells (Fig. 3). Genistein (Fig. 3A) and daidzin (Fig. 3B) aglycones decreased significantly with time and the glucuronide/sulfates appeared. The genistein aglycone level was greater than that of the daidzin aglycone throughout the incubation period (P < 0.05). The amounts of the glucosides, genistin and daidzin, from the cellular extract were stable throughout the incubation period. The intact glucosides, aglycones and their glucuronide/sulfates were all detected in the extract (~0.09 nmol/cm² culture area, 0.003 nmol/cm² culture area and 0.03 nmol/cm² culture area, respec-

**FIGURE 2** Concentrations of genistein (A), daidzin (B), genistin (C) and daidzin (D) and their metabolites in apical solutions after incubation of isoflavones with Caco-2 cells. Isoflavones (10 μmol/L, 6.0 nmol/cm² culture area) were incubated with Caco-2 monolayers for 0.5–2.0 h. The amounts of the glucuronide/sulfates from isoflavone glucosides were just traces and data are not shown in (C) and (D). Results are means ± SD, n = 6–12. Means without a common letter for a compound differ, P < 0.01, and means with * are different from the initial compound at each time point, P < 0.05.

**FIGURE 3** Amounts of genistein aglycone and its glucuronide/sulfates (A) and those of daidzin (B) in cellular extracts after incubation of isoflavones with Caco-2 cells. Isoflavones (10 μmol/L, 6.0 nmol/cm² culture area) were incubated with Caco-2 monolayers for 0.5–2.0 h. Results are means ± SD, n = 6–12. Means without a common letter for a compound differ, $\text{a}$,$\text{b}$ $P < 0.01$, and $\text{c}$,$\text{d}$ $P < 0.05$. *Different from genistein aglycone at each time point.
tively), although the total amounts of isoflavonoids were much less than those of aglycones.

**Amounts of genistein, daidzein, and their glucosides and metabolites in basolateral solution.** Both aglycone and its glucuronide/sulfates were detected in the basolateral solution after incubation of genistein (Fig. 4A) and daidzein (Fig. 4B) with Caco-2 cells. The isoflavone aglycones appeared primarily at the beginning of the incubation period. Interestingly, the glucuronide/sulfates significantly increased with incubation time. Nevertheless, the predominant forms of added genistein and daidzein were intact aglycones throughout the incubation. After 2.0 h of incubation, the amounts of aglycone and glucuronide/sulfates reached ~1.4 nmol/cm² culture area and 0.8 nmol/cm² culture area, respectively. The total recovery of isoflavone aglycone and glucuronide/sulfates corresponded to ~35% of the initial dose of isoflavonoids for both genistein and daidzein.

For genistin and daidzin, intact glucosides, their aglycones and glucuronide/sulfates all appeared in the basolateral solution. The total amounts of isoflavonoids in the basolateral solution were less than one tenth that from their aglycones after 2.0 h incubation (~1.5% of the initial dose, Fig. 4C).

**Estimation of the affinity to the liposomal membrane.** The affinity of each compound toward the cellular membrane indicates the efficiency of cellular uptake of isoflavonoids. PC-LUV suspensions containing isoflavone aglycones or glucosides were separated by ultrafiltration, and unbound isoflavone in the filtered solution was measured. No phospholipid passed through the filter because the average size of the particles (100 nm) was much larger than the pore size (10 nm). Total recovery of isoflavones in the filtrates and the unfiltered fraction was ~80–90%. The concentrations of genistin and daidzin in the filtrates were higher than those of genistein and daidzin (Table 1). The respective concentrations in the filtrate indicated that the affinity toward the liposomal membrane increased in the order of genistin = daidzin < daidzein < genistein.

**Comparison of isoflavonoid and flavonoid levels in the basolateral solution.** To clarify why intact aglycones were predominant in the basolateral solution after the addition of isoflavone aglycones, the transport of genistein was compared with that of flavonoids possessing a B-ring at the 2-position instead of at the 3-position, thus differing from the isoflavonoids (Fig. 1). The flavonoids, quercetin, kaempferol, luteolin and apigenin, were converted efficiently to their glucuronide/sulfates by Caco-2 cells, and the level of the intact aglycone form was significantly less than those of the glucuronide/sulfates in the basolateral solution. This is in contrast to genistein for which the level of the aglycone form exceeded those of the glucuronide/sulfates (Fig. 5).

**FIGURE 4** Amounts of genistein aglycone and its glucuronide/sulfates (A), those of daidzein (B), and genistin, daidzin and their metabolites (C) in basolateral solutions after incubation of isoflavones with Caco-2 cells. Isoflavonoids (10 μmol/L, 6.0 nmol/cm² culture area) were incubated with Caco-2 monolayers for 0.5–2.0 h. The amounts of genistin and daidzin, and their metabolites after 2.0 h incubation are shown in (C). Results are means ± SD, n = 6–12. Means without a common letter for a compound differ, *P < 0.01, and means with # are significantly different from corresponding glucuronide/sulfates, *P < 0.01, #P < 0.05.

**FIGURE 5** Amounts of genistein, flavonoid aglycones and their glucuronide/sulfates in basolateral solutions after 2.0 h incubation with Caco-2 cells. Isoflavonoids/flavonoids (10 μmol/L, 6.0 nmol/cm² culture area) were incubated with Caco-2 monolayers for 2.0 h. Results are means ± SD, n = 3–6. Means of aglycone forms without a common letter differ, *P < 0.01. Means with a and b are significantly different from corresponding glucuronide/sulfates, *P < 0.01, #P < 0.05.
Effect of isoflavonoids and flavonoids on TEER value. The influence of the addition of isoflavonoid to the TEER value of Caco-2 cells was compared with those of flavonoids. Before incubation, the values of TEER for each compound tested were \( \sim 580-700 \ \Omega \cdot \text{cm}^{-2} \). The decreased ratios after 2.0 h incubation were \(< 20\%\) and not different among all compounds tested (data not shown). Thus, as in the flavonoids, paracellular transport was not enhanced by the incubation of isoflavonoid genistein.

Radical scavenging activities of isoflavonoids and flavonoids. The radical scavenging activities were measured as indicators of the redox potential and putative toxicity of the molecules. Quercetin, kaempferol and luteolin showed strong DPPH radical scavenging activity (Table 2). The major compounds in the basolateral solution (Fig. 5) were the glucuronide/sulfates of these three flavonoids. The isoflavone genistein and the flavone apigenin had no radical scavenging activity, although, unlike genistein, the aglycone form of apigenin was inferior to its glucuronide/sulfates in the basolateral solution (Fig. 5). Thus, the radical scavenging activities of the isoflavonoids/flavonoids were not related to their susceptibility to conjugation by Caco-2 cells.

The affinity toward the liposomal membrane of flavonoids. The affinity of flavonoids toward the liposomal membrane was compared with those of isoflavonoids. The flavonoids, quercetin, kaempferol, luteolin and apigenin, had high affinities toward the liposomal membrane because the concentrations of the filtrates, i.e., the fractions unbound to the PC-LUV, were under the detection limit (0.25 μmol/L). Flavonoid aglycones had greater affinities to the membrane than did isoflavonoid aglycones.

DISCUSSION

We reported previously that soy isoflavone aglycones were absorbed faster and in greater quantities than their glucosides in humans (6). Here, intestinal cellular uptake and metabolism of isoflavonoids were investigated using Caco-2 monolayers as an intestinal epithelial model. Earlier, we examined the cellular uptake and metabolism of quercetin and its glucosides by Caco-2 cells and demonstrated that the quercetin aglycone was taken up into Caco-2 cells more efficiently than its glucosides and was converted in large part to glucuronide/sulfates (14). In this study, we found that flavonoid aglycones were also taken up more easily into Caco-2 cells than their glucosides (Figs. 2, 3, 4). This is consistent with a report by Walle et al. (20) that genistein 7-glucoside (genistin) was not transported through the Caco-2 monolayer, probably because the addition of isoflavonoid genistein had greater affinity toward the liposomal membrane because the concentrations of the filtrates, i.e., the fractions unbound to the PC-LUV, were under the detection limit (0.25 μmol/L). Flavonoid aglycones had greater affinities to the membrane than did isoflavonoid aglycones.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radical scavenging activity(^1) (mol DPPH trapped/mol compound)</th>
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</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>1.0</td>
</tr>
<tr>
<td>Genistein</td>
<td>none</td>
</tr>
<tr>
<td>Quercetin</td>
<td>6.56 ± 0.47</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>1.88 ± 0.10</td>
</tr>
<tr>
<td>Luteolin</td>
<td>3.88 ± 0.40</td>
</tr>
<tr>
<td>Apigenin</td>
<td>none</td>
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</tbody>
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\(^1\) Each value is mean ± SD, \( n = 3-6 \).

\(^2\) Amount of DPPH trapped by 1.0 mol of cysteine was assumed to be 1.0 mol.

Furthermore, our in vitro result supports the human study, showing that the absorption of the isoflavone aglycones is superior to that of their glucosides (6). Recently, Andlauer et al. studied the absorption and metabolism of genistein (7) and genistin (8) in isolated rat small intestine and suggested that genistin was absorbed more effectively than genistein. Although these studies considered only genistein, we showed that the other soy isoflavone, daidzein, was also taken up and transported more effectively than daidzin in intestinal Caco-2 monolayers.

Isoflavonoids are present mainly as glycosides in soybeans. Isoflavone aglycones are released from their glycoside in the digestive tract by the hydrolytic action of microlflora (21). We reported previously that β-glucosidase activity is also present in rat intestinal mucosa homogenates (22). Day et al. also found intracellular (23) and membrane-bound β-glucosidase activities (24) in the human small intestine that hydrolyzed genistein-7-glucoside (genistin) to its aglycone. It is therefore likely that hydrolysis enhances the efficiency of intestinal absorption of dietary isoflavonoids. Furthermore, fermented soy foods, in which isoflavone glucuronides are hydrolyzed to isoflavone aglycones during fermentation, may enhance isoflavone intake (5).

We demonstrated that isoflavonoid aglycones were taken up easily as intact aglycone compared with flavonoid aglycones (quercetin, kaempferol, luteolin and apigenin) (Fig. 5). There are two possible pathways by which the intact flavonoids and isoflavonoids could be transported through Caco-2 monolayers. One is that they are transported across the cell membrane without metabolic conversion (the transcellular route). The other is that they pass through the tight junctions bridging the intercellular spaces between the cells (the paracellular route). The effect of isoflavonoids and flavonoids on transepithelial electric resistance (TEER) was measured to clarify the mechanism for the transport of isoflavone aglycones as aglycone forms. Genistein is a potent inhibitor of protein tyrosine kinase (25) and thus may affect the assembly of tight junctions (26). The TEER values did not differ greatly among the compounds, flavonols, flavones and isoflavonoids under the study conditions. We therefore concluded that isoflavonoid aglycones are taken up across the cell membrane and transported without metabolic conversion.

Part of the beneficial effects of flavonoids could come from their antioxidant properties. However, the same flavonoids seem to behave as both antioxidants and prooxidants. The radical scavenging activity reflects both the antioxidant activity and the potential toxicity of the molecule, as indicated by Cao et al. (27). Enterocytes also have conjugation activity (16) to eliminate toxic exogenous molecules. The isoflavonoids, genistein and daidzein, were found to have no radical scavenging activities, although the flavonoids, quercetin, luteolin and kaempferol, had strong activities (Table 2). Interestingly, apigenin, a flavone-type flavonoid, also had no radical scavenging activity, although its major forms in the basolateral solution were glucuronide/sulfates, similar to other flavonoids (Fig. 5). Thus, it is clear that the antioxidant activity and probably the potential toxicity of isoflavonoids and flavonoids are not connected with their susceptibility to conjugation by Caco-2 cells.

Affinity to the cellular membrane seems to play an important role in the efficiency of cellular uptake by the passive diffusion of lipophilic compounds. Our results showed that the affinities of the isoflavone glucosides toward the liposomal membrane were much lower than those of the isoflavone aglycones (Table 1). Isoflavone glucosides were not readily transported through the Caco-2 monolayer, probably because...
of their lack of affinity toward the lipid bilayer of the cell surface. Of the isoflavone aglycones, genistein possesses a stronger affinity toward the liposomal membrane than daidzein. This reflects the greater incorporation of the aglycone genistein than daidzein into the cellular extract. On the other hand, both isoflavone aglycones possess lower affinity than flavones (quercetin and kaempferol) and flavonoids (apigenin and luteolin). This might explain the difference in cellular uptake efficiency between isoflavonoids and flavonoids.

There are several reports on the structures of dietary flavonoids present in plasma, in both intact and conjugated metabolite forms. We demonstrated earlier that no aglycone form was detected in rat (28) or human plasma (29) when quercetin or a quercetin-rich diet was administered. Other research groups have also indicated that the major forms in rat (30) and human plasma (31) are quercetin glucuronide/sulfate conjugates. It is noteworthy that Adlercreutz et al. (32) and Barnes et al. (33) have also suggested the predominance of glucuronide/sulfate isoflavonoids in human plasma after intake of soy products, as noted in this study. On the other hand, it was reported that isoflavone aglycones were present in human plasma when subjects ingested soy supplements (34). Holder et al. (35) also reported that some isoflavone aglycones were present in intact form in the plasma of rats fed a genistein-containing diet. If isoflavone aglycones are easily transported across the intestinal epithelium in an intact form, most of them should be metabolized in the liver. Nevertheless, when intact aglycones are absorbed efficiently, a part of the intact aglycone, a biologically active form, may be transported through intestinal cells into the circulation without further metabolism. Our previous results identified quercetin 3-glucuronide/sulfate as one of the major quercetin metabolites in rat plasma (28). Isoflavonoids possess a B-ring at the 3-position instead of the 2-position, thus differing from the flavonoids including quercetin (Fig. 1). It is unlikely that the intracellular conjugation enzyme in the small intestine, uridine 5'-diphosphoglucuronosyltransferase (16), effectively converts isoflavone aglycones to their glucuronide/sulfates.

In conclusion, the soy isoflavone aglycones, genistein and daidzein, are taken up more efficiently into enterocytes than their glucosides in humans. J. Nutr. 130: 843–846.