Enzymatically Modified Isoquercitrin, α-Oligoglucosyl Quercetin 3-*O*-Glucoside, Is Absorbed More Easily than Other Quercetin Glycosides or Aglycone after Oral Administration in Rats

Toshiaki Makino,*[,a] Ryosuke Shimizu,[b] Misaki Kanemaru,[a] Yukio Suzuki,[b] Masamitsu Moriwaki,[b] and Hajime Mizukami[a]

[a] *Department of Pharmacognosy, Graduate School of Pharmaceutical Science, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan: and* [b] *San-Ei-Gen F.F.I., Inc.; 1–1–11 Sanwa-cho, Toyonaka, Osaka 561–8588, Japan.* Received July 27, 2009; accepted October 2, 2009; published online October 5, 2009

Quercetin, a flavonol contained in various vegetables and herbal medicines, has various biological activities including anti-cancer, anti-allergic and anti-oxidative activities. However, low oral bioavailability of quercetin due to insolubility in water has limited its use as a food additive or dietary supplement. Since the water solubility is enhanced by glycosyl conjugation, in the present study, we evaluated the bioavailability of several quercetin glycosides with different sugar moieties in rats. Quercetin, quercetin-3-*O*-rutinoside (rutin), and quercetin-3-*O*-glucoside (isoquercitrin, IQC) in suspension, and quercetin-3-*O*-maltoside (Q3M), quercetin-3-*O*-gentiobioside (Q3G), α-monoglucosyl rutin (αMR), α-oligoglucosyl rutin (αOR), and enzymatically modified isoquercitrin (α-oligoglucosyl isoquercitrin, EMIQ) dissolved in water, were orally administered to rats under anesthesia. Bioavailability (*F* value) was calculated from the concentrations of total quercetin in plasma from 0 to 12 h after the administration. *F* value of quercetin was 2.0%, and those of IQC, Q3M and EMIQ were 12%, 30%, and 35%, respectively. Although Q3G, αMR and αOR have high water solubility, their *F* values were low (3.0%, 4.1%, 1.8%, respectively). In the *in vitro* study, the homogenate of rat intestinal epithelium rapidly hydrolyzed IQC, Q3M and EMIQ to quercetin, and αMR and αOR to rutin. However, it could not hydrolyze Q3G or rutin to quercetin. Elongation of α-linkage of glucose moiety in IQC enhances the bioavailability of quercetin, and intestinal epithelial enzymes such as lactase-phrolizin hydrolase or mucosal maltase-glucoamylase would play important roles in the hydrolysis and absorption of these flavonol glycosides.

Key words enzymatically modified isoquercitrin; quercetin; rutin; bioavailability; lactase-phlorizin hydrolase

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a widely distributed secondary metabolite in plants. Human beings regularly consume quercetin in various fruits, vegetables or herbal medicines; such as apple, onion, and sophora flower. Quercetin has been found to have various biological activities including antiproliferative effects on several cancer cells,[1—3] anti-inflammatory and anti-allergic effects,[4] anti-oxidative activity,[5] and atherosclerosis-preventing effect.[6] In clinical study, supplementation of quercetin in hypertensive patients for 28 d significantly reduced blood pressure.[7] However, orally administered quercetin is poorly absorbed, and the bioavailability of quercetin administered in capsule form to human beings was reported to be less than 1%.[8] Therefore, several trials have been conducted to increase the bioavailability of quercetin by optimizing its formulation. For example, a pharmacokinetic study in rats revealed that bioavailability of quercetin was increased 5.7-fold by administration using a solid lipid nanoparticle as an oral delivery carrier compared with that administered as a quercetin suspension.[9]

Quercetin is generally accumulated in plants as glycosides such as glucosides, rutinosides and xylosides. These quercetin glycosides show higher solubility in water than quercetin due to the hydrophilicity of the sugar moieties. Indeed, quercetin-4′-*O*-β-D-glucopyranoside has higher bioavailability than its aglycone in human beings, suggesting that conjugation with glucose would enhance quercetin absorption in the small intestine.[10,11]

Glucosyl conjugation of lipophilic small molecules leads to an increase in their water solubility.[12] Several glucosides of quercetin have been prepared by enzymatic synthesis to enhance the water solubility, as exemplified by α-monoglucosyl rutin (αMR),[13] α-oligoglucosyl rutin (αOR),[14] and "enzymatically modified isoquercitrin" (EMIQ).[15] In Japan, EMIQ has been approved as a food additive,[16] and in U.S.A., FDA declared EMIQ generally regarded as safe (GRAS) for use in multiple food categories. However, how glucosyl conjugation affects the pharmacokinetic behavior of quercetin remains unknown except that αMR exhibited 4.5-fold higher area under the plasma concentration–time curve (*AUC*) value than quercetin after oral administration as a suspension in carboxymethylcellulose (CMC) solution in rats.[13]

In the present investigation, we examined the intestinal absorption and bioavailability of quercetin after oral administration of various quercetin glucosides, and revealed that glucosyl conjugation of isoquercitrin (IQC) with α-glucosidic linkages is quite effective for improving the bioavailability. We also showed that hydrolysis of the glycosidic linkage prior to the absorption through small intestinal epithelium is essential. We further suggest that the collaborative action of two glucosidases, mucosal maltose-glucoamylase (MGAM) and lactase-phlorozin hydrolase (LPH), both anchoring in the mucosal membrane of the epithelial tissue, plays a pivotal role in absorption of quercetin glucosides in the small intestine.

MATERIALS AND METHODS

Chemicals Quercetin and rutin were purchased from Tokyo Chemical Industry (Tokyo, Japan), and α-monoglucosyl rutin (αMR) from Wako Pure Chemical Industries

* To whom correspondence should be addressed. e-mail: makino@phar.nagoya-cu.ac.jp

(Osaka). Isoquercitrin (quercetin 3-*O*-glucoside, IQC), "enzymatically modified isoquercitrin" (EMIQ) and α-oligoglucosyl rutin (αOR) were prepared by San-Ei-Gen F.F.I. (Osaka, Japan).[14,15] Quercetin 3-*O*-maltoside (Q3M) and quercetin 3-*O*-gentiobioside (Q3G) were from our laboratory stock. Figure 1 shows the chemical structures of these compounds. Purities of quercetin, rutin, IQC, Q3M, and Q3G were estimated to be 85.4, 92.0, 91.6, 84.9 and 93.9% (w/w), respectively. αMR contains 13.4% (w/w) of IQC as an impurity according to the previous study.[13] EMIQ and αOR were the mixtures of glucosides of IQC and rutin, respectively. The average molecular weight was estimated to be 783 for EMIQ and 1313 for αOR by the manufacturer. Isorhamnetin, tamarixetin (Fig. 1, respectively) and diosmetin were purchased from Extrasynthase (Genay Cedex, France). All other chemicals were of commercial reagent grade quality.

Animal Experiments Experimental procedures were approved by the Animal Care Committee at Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, in accordance with the guidelines of the Japanese Council on Animal Care. Male Wistar ST rats were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a temperature-controlled room (at 23±1 °C) with lighting from 7 a.m. to 7 p.m. and freely accessed to a commercial standard diet (CE-2, Oriental yeast, Tokyo) and water under conventional conditions.

EMIQ, Q3M, Q3G, αMR and αOR are practically freely soluble in water. Water solubilities of quercetin, IQC and rutin were estimated to be 50.0, 206 and 196 nmol/ml, respectively. EMIQ was dissolved in distilled water at a concentration of 0.31, 1.1 and 5.0 mM. Q3M, Q3G, αMR and αOR were dissolved in distilled water at a concentration of 5.0 mM. Quercetin, rutin, and IQC were suspended in distilled water at concentration of 5.0 mM by sonication for 30 min. The suspensions were prepared just before the experiments. To wash out the flavonoids in the body, we fed 9-week-old rats AIN-76 (Clea Japan Inc., Tokyo) that is flavonoid-free for 1 week. Then, the rats (n=6 or 7) without fasting were intraperitoneally injected with 1.0 g/kg of urethane (Sigma-Aldrich, St. Louis, MO, U.S.A.) for deep anesthesia, and sample solutions or suspensions (10 ml/kg) were orally administered by gavage. In a separate experiment, quercetin was dissolved in dimethylsulfoxide at a concentration of 1.0 M, and intravenously injected into jugular vein (50 μl/kg) of the rats (n=6). Blood (0.25 ml) was collected *via* either side of jugular veins alternately at 0.25, 0.5, 1, 2, 3, 6, 9 and 12 h after the oral or intravenous administration into heparin-coated tubes. The blood was centrifuged (12000 ***g***,

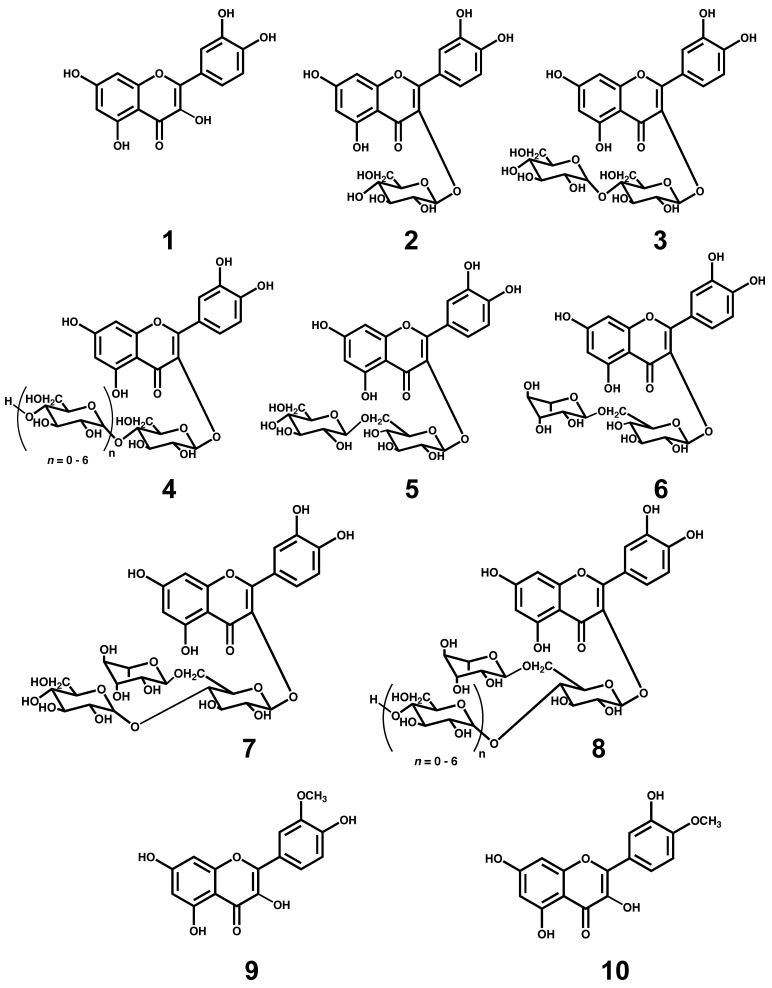

Fig. 1. Chemical Structures of Quercetin (**1**), Quercetin-3-*O*-glucoside (Isoquercitrin, IQC, **2**), Quercetin-3-*O*-maltoside (Q3M, **3**), Enzymatically Modified Isoquercitrin (EMIQ, **4**), Quercetin-3-*O*-gentiobioside (Q3G, **5**), Rutin (**6**), α-Monoglucosyl Rutin (αMR, **7**), α-Oligoglucosyl Rutin (αOR, **8**), Isorhamnetin (**9**) and Tamarixetin (**10**)

7 min, 4 °C) to obtain plasma, which was stored at −80 °C until analysis.

HPLC Analysis of Plasma Concentration of Quercetin and Its Metabolites A 90 μl aliquot of plasma was mixed with 20 μl of β-glucuronidase from *Helix pomatia* (Sigma-Aldrich #G0751, 2.9×10^4 units/ml of β-glucuronidase and $>5\times10^2$ units/ml of sulfatase) in 0.58 M acetate buffer, pH 4.9, and incubated at 37 °C for 60 min. Then 25 μl of 10 mM sodium metaphosphate solution containing 1 M L-ascorbic acid and 9 μl of diosmetin (20 μg/ml) was added to the reaction mixture, which was then lyophilized. The lyophilizate was dissolved in 1 ml of MeOH, and after centrifugation (14000 ***g***, 10 min), the supernatant was transferred into a fresh tube and dried up under nitrogen flow. The residue was dissolved in 180 μl of MeOH/200 mM HCl (1 : 1) solution, and after centrifugation (14000 ***g*** for 7 min) the supernatant (100 μl) was subjected to HPLC analysis under the following conditions: system (1100 series, Agilent, CA, U.S.A.); column, YMC Pack ODS-AQ (4.6×250 mm, YMC Co., Ltd., Kyoto, Japan); mobile phase, 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B) (B/A=25/75—35/65 for 5 min; 35/65 for 5—20 min; 100/0 for 20—30 min; 25/75 for 30—37 min, linear gradient); flow rate, 1.0 ml/min; column-temperature, 30 °C; detection, absorbance at 370 nm; retention times, quercetin, 14.2 min; isorhamnetin, 22.0 min; tamarixetin, 22.5 min; diosmetin, 20.8 min. Linear regression of the concentration range of 0.132—3.64 μM for quercetin, 0.221—8.75 μM for isorhamnetin, 0.095—1.58 μM for tamarixetin by the peak-area ratio of these compounds to diosmetin was calibrated by the least-squares method ($r^2=0.99$).

Pharmacokinetic Analysis The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) of total quercetin were obtained from the actual data observed after oral administration. *AUC* from 0 to 12 h after administration ($AUC_{0—12\,h}$) was calculated using the trapezoidal rule from the first measurement point to the last measurement point. Bioavailability (*F* value) was calculated by dividing $AUC_{0—12\,h}$ after the oral administration of the samples by the $AUC_{0—12\,h}$ after the intravenous administration of quercetin. Data were expressed as mean±S.E. ($n=6$—7). Significant differences among the groups were determined by one-way ANOVA followed by Bonferroni/Dunn multiple comparison test using Statcel2®.[17] *p* values less than 0.05 were considered statistically significant.

In Vitro* Enzymatic Digestion of Quercetin Glycosides** The upper third of small intestine was collected from male Wistar/ST rats (9 weeks old). After removal of the contents by perfusing with ice-cold 100 mM citrate buffer (pH 4.8), the intestine was cut lengthwise, and epithelial tissue was stripped with a spatula. The epithelium was homogenized in the citrate buffer by sonication for 1 min on ice. After centrifugation (14000 ***g, 10 min), the supernatant was used as a crude epithelial enzyme. The protein concentration in the supernatant was measured using a BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL, U.S.A.). Quercetin glycosides were dissolved at a concentration of 100 μM in the citrate buffer, and a 750 μl aliquot of glycoside solution was mixed with the same volume of the crude epithelial enzyme. The mixture was incubated at 37 °C, and a 100 μl aliquot of the reactant was collected at an appropriate time. The reaction was stopped by adding 400 μl of EtOH containing baicalein (50 μM). The mixture was subjected into HPLC analysis.

For the analysis of the reaction products from EMIQ, Q3M, Q3G, and IQC, LC/MS/MS system (Waters Quattro Premier XE, Milford, MA, U.S.A.) with an electrospray ionization source in the negative ion mode with multiple reaction monitoring was used. The analytical column was a Cadenza CD-C18, 50×2.1 mm, 3 μm (Imtakt Inc., Kyoto). The mobile phase was delivered by a linear gradient elution system, 0.1% AcOH in H_2O (solvent A) : 0.1% AcOH in acetonitrile (solvent B) (B/A=14/86—65/35 for 2.5 min; 65/35—95/5 for 2.5—3.0 min; 95/5—100/0 for 3.0—5.0 min; 100/0 for 5.0—6.0 min) at a flow rate of 200 μl/min. Injection volume of the sample was 10 μl. Both quadrupoles were maintained at the unit resolution and the transitions (precursor to daughter) monitored were *m*/*z* 625.5 to 300.1 for Q3M (retention time, 3.3 min) and Q3G (3.4 min), *m*/*z* 463.0 to 301.0 for IQC (3.6 min), *m*/*z* 300.9 to 178.8 for quercetin (4.2 min), and *m*/*z* 268.9 to 251.0 for baicalein (4.4 min). Linear regression in a concentration range of 0.67—54 μM for Q3M, IQC and quercetin, and 18—54 μM for Q3G was calibrated by the peak-area ratio of these compounds to baicalein by the least-squares method ($r^2>0.99$).

For the analysis of the enzymatic reaction products from rutin, αMR, and αOR, HPLC/UV system (LC-10A$_{VP}$, Shimadzu, Kyoto, Japan) with a photodiode array detector (SPD-10A, Shimadzu) was used. The analytical column was an Inertosil ODS-3 (4.6×250 mm, GL Science, Tokyo). The mobile phase was delivered with a linear gradient elution system, 0.1% AcOH in H_2O (solvent A) : 0.1% AcOH in MeOH (solvent B), (B/A=40/60 for 10 min; 40/60—96/4 for 10—11 min; 96/4—100/0 for 11—16 min; 100/0 for 16—20 min) at a flow rate of 1.0 ml/min. Injection volume of the sample was 10 μl. The UV detector was set at 254 nm. Linear regression in a concentration range of 2.0—54 μM for rutin (retention time, 9.4 min) and αMR (9.1 min), 2.0—18 μM for IQC (9.9 min) and quercetin (15.8 min) was calibrated by the peak-area ratio of these compounds to baicalein (16.8 min) with the least-squares method ($r^2=0.99$).

Data were compensated by the concentration of proteins in the reaction solution, and expressed as mean±S.D. ($n=3$).

RESULTS

Oral administration of EMIQ at a dose of 50 μmol/kg led to a rapid increase in plasma concentration of quercetin and its methylated metabolite, tamarixetin (Figs. 2A, C). The plasma level reached a maximum 15 min for quercetin and 15—30 min for tamarixetin after the administration. The concentration of quercetin gradually decreased while that of tamarixetin remained at a plateau thereafter. The plasma concentration of isorhamnetin, another methylated metabolite of quercetin, gradually increased and reached a peak 6 h after administration, that remained at the peak level thereafter (Fig. 2B). Plasma concentrations of quercetin and its methylated metabolites were depended on the EMIQ doses (3.1, 11, 50 μmol/kg), and no saturation was observed in C_{max} and $AUC_{0—12\,h}$ values within the range of EMIQ doses examined (Table 1).

The plasma concentrations of quercetin and its metabolites after oral administration (50 μmol/kg) of Q3M and IQC

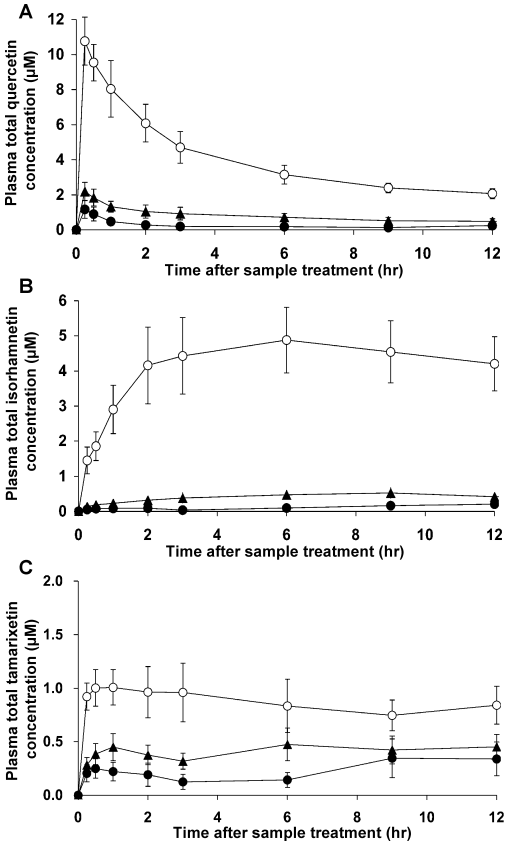

Fig. 2. Pharmacokinetic Profile of Quercetin (A), Isorhamnetin (B) and Tamaxiretin (C) after Oral Administration of EMIQ in Rats

EMIQ was dissolved in H_2O and the solutions were orally administrered to rats at the dosage of 50 (open circles), 11 (closed triangles) and 3.1 (closed circles) μmol/kg. Plasma samples were treated with β-glucuronidase/sulfatase, and the plasma concentrations of quercetin and its metabolites were measured. Data were expressed as mean±S.E. (n=6).

Table 1. Pharmacokinetic Parameters of Quercetin and Its Metabolites after Oral Adminstration of EMIQ in Rats

EMIQ (μmol/kg)	Quercetin		Isorhamnetin	Tamarixetin
	C_{max} (μM)	$AUC_{0—12\,h}$ (μM·h)	$AUC_{0—12\,h}$ (μM·h)	$AUC_{0—12\,h}$ (μM·h)
3.1	1.23±0.49	2.80±0.51	1.43±0.29	2.89±1.41
11	2.55±0.48	9.29±2.60	5.34±1.64	5.29±1.44
50	10.7±1.5	46.0±7.0	55.1±11.3	11.2±2.4

EMIQ was dissolved in H_2O and the solutions were orally administered. Plasma samples were treated with β-glucuronidase/sulfatase, and the concentrations of quercetin and its metabolites were measured. The *AUC* was calculated by the trapezoid rule, and data were expressed as mean±S.E. (n=6).

showed a change similar to that after EMIQ administration, but the plasma levels were about 80% and 30% for Q3M and IQC, respectively, of those for EMIQ (Fig. 3). The plasma concentrations of quercetin and its metabolites showed only a slight increase after oral administration of αMR, αOR and Q3G, and only a trace level after administration of quercetin and rutin (Fig. 3). Pharmacokinetic parameters of quercetin and its methylated metabolites were examined (Table 2). C_{max}, $AUC_{0—12\,h}$ and F values of quercetin were significantly higher when EMIQ or Q3M was administered than those when other quercetin glycosides were administered. $AUC_{0—12\,h}$ values of isorhamnetin and tamarixetin were paralleled to

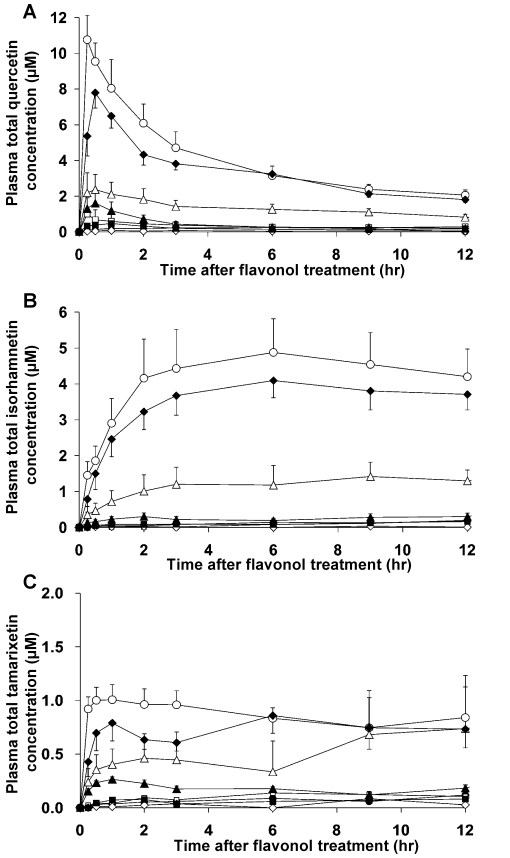

Fig. 3. Pharmacokinetic Profile of Quercetin (A), Isorhamnetin (B) and Tamaxiretin (C) after Oral Administration of Flavonol Glycosides in Rats

EMIQ (open circles), Q3M (closed diamonds), IQC (open triangles), αRM (closed triangles), Q3G (open squares), αOR (closed squares), quercetin (closed circle) and rutin (open diamonds) was dissolved or suspended in H_2O (5 mM), and the solutions were orally administered to rats at the dosage of 50 μmol/kg. Plasma samples were treated with β-glucuronidase/sulfatase, and the plasma concentrations of quercetin and its metabolites were measured. Data were expressed as mean±S.E. (n=6—7).

that of quercetin.

We examined the enzymatic hydrolysis of quercetin glucosides using the crude extract prepared from rat intestinal epithelium. EMIQ was rapidly converted to quercetin during incubation with the crude enzyme. The transient increase in IQC concentration indicated that IQC was an intermediate of the EMIQ digestion (Fig. 4A). In fact, IQC was readily hydrolyzed to quercetin (Fig. 4B). Q3M was also hydrolyzed to quercetin *via* IQC by the crude enzyme preparation (Fig. 4C). αMR was rapidly digested to rutin (Fig. 4D), and αOR was converted to αMR and then to rutin (Fig. 4E). However, in either case no further conversion of rutin to IQC or quercetin was observed. Rutin itself was not hydrolyzed at all by the crude enzyme preparation from small intestinal epithelium (Fig. 4F). Q3G was slowly converted to quercetin although the formation of IQC as a reaction intermediate was not detected, and only 7.6% of the initial Q3G was hydrolyzed to quercetin after a 2-h incubation (Fig. 4G).

DISCUSSION

Orally administered quercetin is poorly absorbed.[8,18] The bioavailability of quercetin is improved by dissolving in a vehicle containing an organic solvent. For example, quercetin dissolved in dimethylsulfoxide/polyethylene glycol 200 has

Table 2. Pharmacokinetic Parameters of Quercetin and Its Metabolites after Oral Administration of Several Flavonoid Glycosides in Rats

	Quercetin			Isorhamnetin	Tamarixetin
	C_{max} (μM)	$AUC_{0—12\,h}$ (μM·h)	F value (%)	$AUC_{0—12\,h}$ (μM·h)	$AUC_{0—12\,h}$ (μM·h)
EMIQ	$10.7 \pm 1.5^{a)}$	$46.0 \pm 7.0^{a)}$	$35.0 \pm 5.3^{a)}$	$55.1 \pm 11.3^{a)}$	$11.2 \pm 2.4^{a,c,d)}$
IQC	$2.66 \pm 0.81^{b)}$	$15.8 \pm 3.6^{b)}$	$12.1 \pm 2.8^{b)}$	$13.8 \pm 4.6^{b)}$	$6.70 \pm 2.81^{a,b,c,d)}$
Quercetin	$0.26 \pm 0.06^{b)}$	$2.6 \pm 0.7^{b)}$	$2.0 \pm 0.5^{b)}$	$1.50 \pm 4.59^{b)}$	$0.89 \pm 0.23^{b,d)}$
Q3M	$8.36 \pm 1.30^{a)}$	$39.4 \pm 4.8^{a)}$	$30.0 \pm 3.7^{a)}$	$42.9 \pm 0.1^{a)}$	$8.51 \pm 1.60^{a,c)}$
Q3G	$0.91 \pm 0.62^{b)}$	$3.9 \pm 1.6^{b)}$	$3.0 \pm 1.2^{b)}$	$0.91 \pm 0.13^{b)}$	$1.19 \pm 0.33^{b,d)}$
Rutin	$0.11 \pm 0.02^{b)}$	$1.0 \pm 0.4^{b)}$	$0.8 \pm 0.3^{b)}$	$0.79 \pm 0.61^{b)}$	$0.60 \pm 0.30^{b,d)}$
αMR	$1.87 \pm 0.76^{b)}$	$5.3 \pm 1.3^{b)}$	$4.1 \pm 1.0^{b)}$	$3.10 \pm 0.97^{b)}$	$2.10 \pm 0.61^{b,c,d)}$
αOR	$0.69 \pm 0.21^{b)}$	$2.4 \pm 0.4^{b)}$	$1.8 \pm 0.3^{b)}$	$1.25 \pm 0.28^{b)}$	$0.72 \pm 0.20^{b,d)}$

Each quercetin glycoside was dissolved or suspended in H_2O (5 mM), and administered to rats at the dosage of 50 μmol/kg. Plasma samples were treated with β-glucuronidase/sulfatase, and the concentrations of quercetin and its metabolites were measured. The $AUC_{0—12\,h}$ values were calculated by the trapezoid rule, and F values were calculated as the ratios of AUC to the mean of $AUC_{0—12\,h}$ values when quercetin dissolved in DMSO was injected into the vein (131 ± 6 μM·h, $n=5$). Data were expressed as mean±S.E. ($n=6$—7). *a*—*d*) Values with different letters are significantly different at the levels of $p<0.05$ by Bonferroni/Dunn multiple comparison tests.

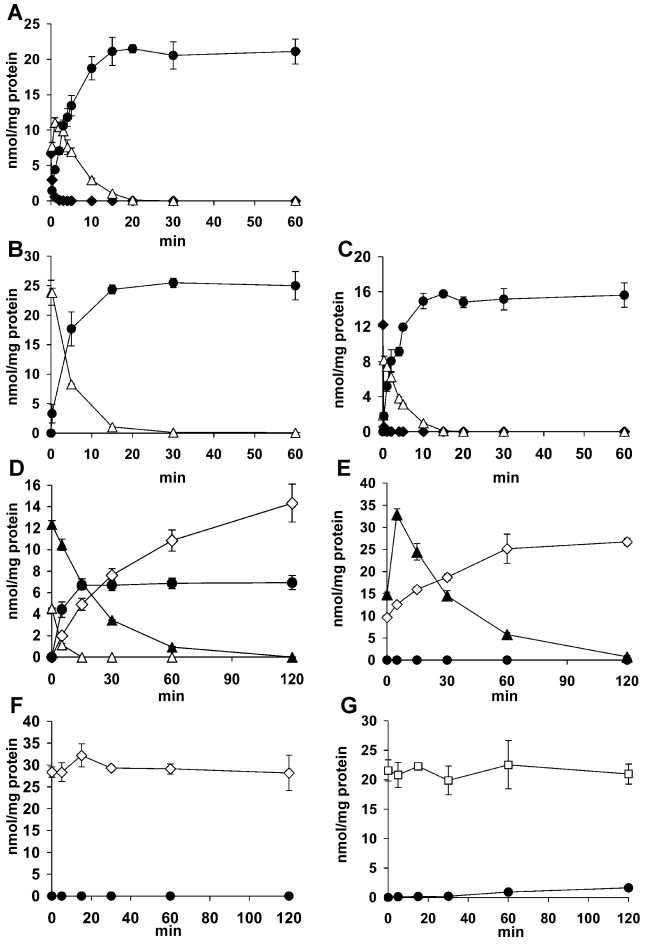

Fig. 4. Hydrolysis of EMIQ (A), IQC (B), Q3M (C), αRM (D) and αOR (E), Rutin (F), Q3G (G) by the Homogenate of Rat Intestinal Epithelium

Each sample was incubated with the homogenate at 37 °C, and the concentrations of quercetin (closed circles), IQC (open triangles), Q3M (closed diamonds), Q3G (open squares), rutin (open diamonds) and αRM (closed triangles) in the reactant were measured. The data were calibrated by the concentration of proteins in the reactant, and expressed as mean±S.D. ($n=3$).

up to 1.3-fold bioavailability in rats compared with that suspended in 0.7% CMC solution.[19] In our preliminary experiment, the bioavailability of quercetin dissolved in dimethylsulfoxide/water (70 : 30) solution in rats was 3.2-fold higher than that of quercetin suspended in water. Thus, the solubility of quercetin is a highly important factor for its bioavailability.

In the present study, we compared the pharmacokinetic parameters of the glucosyl conjugates of quercetin and rutin with enhanced water solubility. Of the five glucosyl conjugates examined, EMIQ and Q3M exhibited drastic increase in C_{max} and bioavailability of quercetin. Although the result was obtained using anesthetized rats, increase in the *AUC* value of EMIQ was also reported previously using conscious rats (Ono *et al.* 2005). IQC revealed the increase in these parameters, but to a less extent. These results may reflect the participation of water solubility in the bioavailability of these glucosides because EMIQ and Q3M were readily dissolved in water at 5 mM concentration whereas IQC was not completely dissolved in water at this concentration. In contrast, the intestinal absorption of Q3G was poor although it is as soluble in water as Q3M. These results suggest that not only water solubility but also chemical structure of the glucose moiety of a particular glycoside affects the intestinal absorption of quercetin glycoside, because an additional glucose molecule is conjugated to IQC with an α-(1,4)-linkage and a β-(1,6)-linkage in Q3M and Q3G, respectively. The absorption of αMR and αOR was only slightly improved compared with that of rutin although they are quite soluble in water.

Quercetin and its methylated metabolites were observed in plasma as early as 15 min after oral administration of EMIQ and Q3M, and T_{max} values of quercetin were 15 and 30 min for EMIQ and Q3M, respectively. Thus, it is unlikely that these compounds were absorbed through the large intestine after they were digested into the aglycone by intestinal microflorae, especially because the intestinal movement is supposed to be suppressed by anesthesia with urethane in the present investigation.[20] In fact, recent clinical studies indicated that the primary site of absorption of dietary glycosides was small intestine.[10,21,22] Two possible mechanisms, involvement of glucose transporters and deglycosylation by glycosidase in small intestine, can explain these observations. However, sodium-dependent glucose transporter-1 (SGLT1) has recently been found not to be involved in quercetin glycoside absorption.[23] There are evidences that deglycosylation is prerequisite for absorption of dietary flavonoid glycosides through the small intestine and that quercetin aglycone can be transported through highly lipophilic epithelial membrane.[24]

Various glucosidases are present in the small intestine,

including glucocerobrosidase, lactase-phlorizin hydrolase (LPH), cytosolic β-glucosidase (CBG), and pyridoxine glucoside hydrolase. Among them LPH is a sole β-glucosidase present on the luminal side of the brush border of small intestine and contributes to deglucosylation of the dietary flavonoid glucosides prior to the absorption.[24] Mucosal maltose-glucoamylase (MGAM) with hydrolytic activity on α-(1,4)-glucosidic linkage is also present in the small intestine, anchoring to the brush border epithelial cells, and is involved in the final step in small intestinal starch digestion.[25] We prepared crude extract from epithelial cells of rat small intestine and examined the hydrolysis of quercetin glycosides *in vitro*. IQC was rapidly converted to quercetin by incubation with the crude enzyme. This reaction is catalyzed by LPH, since CBG did not hydrolyze IQC[26] while purified LPH was shown to efficiently digest IQC to quercetin.[24] Since LPH is a transmembrane-type protein locating at the luminal side of brush border membrane of intestinal epithelial cells,[27] quercetin aglycone would soon dissolve into lipophilic membrane, and penetrate into the intestine by simple diffusion. The changes in the enzymatic hydrolysis of EMIQ and Q3M with the lapse of time clearly indicated that these glucosides were first converted to IQC, which was then rapidly converted to quercetin. The first step is an enzymatic hydrolysis of α-(1,4)-glucosidic linkage presumably catalyzed by MGAM. MGAM may also be involved in α-glucosidic hydrolysis of αMR and αOR, which results in accumulation of rutin in the reaction mixtures. Rutin was not hydrolyzed to quercetin by the crude enzyme preparation presumably because no enzyme capable of hydrolyzing the α-rhamnosyl linkage is present in the epithelium of small intestine. This is consistent with the results that the bioavailability of neither αMR nor αOR was increased compared with quercetin in the present investigation. Shimoi *et al.* reported that the $AUC_{0-24\,h}$ for quercetin in rats administered αMR was 4.5-fold higher than that in rats administered quercetin.[13] Since they measured the plasma concentration of quercetin and its metabolites in awake rats for a longer period than the present investigation, their results might reflect not the absorption in the small intestine but the microflorae digestion of rutin in the large intestine. In addition, they used 0.5% CMC solution, which might promote the absorption of these compounds, as a vehicle of quercetin and αMR, although we used water.

In this study, we also measured plasma concentarations of methylated quercetin, *i.e.* isorhamnetin, and tamarixetin. The plasma concentration of isorhamnetin, a major methylated metabolite, gradually increased by 2 h after administration and remained at the peak level thereafter. Thus it is unlikely that quercetin is at small intestine prior to transfer into the circulation. In fact, catechol-*O*-methyltransferase is much more abundant in liver and kidney than intestine in rats.[28]

In conclusion, glucosyl conjugation is an effective strategy to improve the bioavailability of quercetin. Quercetin glucosides should be hydrolyzed to quercetin prior to absorption in small intestine. Since α-(1,4)-glucosyl linkage is susceptible to digestion by MGAM and resultant IQC is readily converted to quercetin by LPH, conjugation of glucose residues to IQC with α-(1,4)-linkage is a preferred approach to improve the quercetin absorption. The present study revealed that EMIQ exhibits the highest bioavailability among the glycosides examined in rats. This may contribute to the potent pharmacological activities of EMIQ, including anti-hypertensive effects in spontaneous hypertensive rats,[29] atheroprotective and plaque-stabilizing effects in ApoE-deficient mice,[30] and suppressive effects on the symptoms in cedar pollinosis patients.[31] Comparison of pharmacological activities among these quercetin glycosides is now in progress.

Acknowledgment This work was supported from the Japan Food Chemical Research Foundation (2008).

REFERENCES

1) Jeong J. H., An J. Y., Kwon Y. T., Rhee J. G., Lee Y. J., *J. Cell. Biochem.*, **106**, 73—82 (2009).
2) Luo H., Jiang B. H., King S. M., Chen Y. C., *Nutr. Cancer*, **60**, 800—809 (2008).
3) Choi E. J., Bae S. M., Ahn W. S., *Arch. Pharm. Res.*, **31**, 1281—1285 (2008).
4) Park H. H., Lee S., Son H. Y., Park S. B., Kim M. S., Choi E. J., Singh T. S., Ha J. H., Lee M. G., Kim J. E., Hyun M. C., Kwon T. K., Kim Y. H., Kim S. H., *Arch. Pharm. Res.*, **31**, 1303—1311 (2008).
5) Kim S. K., Kim H. J., Choi S. E., Park K. H., Choi H. K., Lee M. W., *Arch. Pharm. Res.*, **31**, 424—428 (2008).
6) Hayek T., Fuhrman B., Vaya J., Rosenblat M., Belinky P., Coleman R., Elis A., Aviram M., *Arterioscler. Thromb. Vasc. Biol.*, **17**, 2744—2752 (1997).
7) Edwards R. L., Lyon T., Litwin S. E., Rabovsky A., Symons J. D., Jalili T., *J. Nutr.*, **137**, 2405—2411 (2007).
8) Gugler R., Leschik M., Dengler H. J., *Eur. J. Clin. Pharmacol.*, **9**, 229—234 (1975).
9) Li H., Zhao X., Ma Y., Zhai G., Li L., Lou H., *J. Controlled Release*, **133**, 238—244 (2008).
10) Hollman P. C., de Vries J. H., van Leeuwen S. D., Mengelers M. J., Katan M. B., *Am. J. Clin. Nutr.*, **62**, 1276—1282 (1995).
11) Hollman P. C. H., van Trijp J. M. P., Buysman M. N. C. P., v.d. Gaag M. S., Mengelers M. J. B., de Vries J. H. M., Katan M. B., *FEBS Lett.*, **418**, 152—156 (1997).
12) Kaminaga Y., Nagatsu A., Akiyama T., Sugimoto N., Yamazaki T., Maitani T., Mizukami H., *FEBS Lett.*, **555**, 311—316 (2003).
13) Shimoi K., Yoshizumi K., Kido T., Usui Y., Yumoto T., *J. Agric. Food Chem.*, **51**, 2785—2789 (2003).
14) Suzuki Y., Suzuki K., *Agric. Biol. Chem.*, **55**, 181—187 (1991).
15) Akiyama T., Washino T., Yamada T., Koda T., Maitani T., *J. Food Hyg. Soc. Jpn.*, **41**, 54—60 (2000).
16) Japan Food Additives Association, "Japanese Specifications and Standards for Food Additives," 8th ed., Japan Food Additives Association, Tokyo, 2007.
17) Yanai S., "4 Steps Excel Statistics," 2nd ed., OMS Publishers, Tokorozawa, 2004.
18) Piskula M., Terao J., *J. Agric. Food Chem.*, **46**, 4313—4317 (1998).
19) Khaled K. A., El-Sayed Y. M., Al-Hadiya B. M., *Drug Dev. Ind. Pharm.*, **29**, 397—403 (2003).
20) Yuasa H., Matsuda K., Watanabe J., *Pharm. Res.*, **10**, 884—888 (1993).
21) Erlund I., Kosonen T., Alfthan G., Maenpaa J., Perttunen K., Kenraali J., Parantainen J., Aro A., *Eur. J. Clin. Pharmacol.*, **56**, 545—553 (2000).
22) Graefe E. U., Wittig J., Mueller S., Riethling A. K., Uehleke B., Drewelow B., Pforte H., Jacobasch G., Derendorf H., Veit M., *J. Clin. Pharmacol.*, **41**, 492—499 (2001).
23) Kottra G., Daniel H., *J. Pharmacol. Exp. Ther.*, **322**, 829—835 (2007).
24) Nemeth K., Plumb G. W., Berrin J. G., Juge N., Jacob R., Naim H. Y., Williamson G., Swallow D. M., Kroon P. A., *Eur. J. Nutr.*, **42**, 29—42 (2003).
25) Ao Z., Quezada-Calvillo R., Sim L., Nichols B. L., Rose D. R., Sterchi E. E., Hamaker B. R., *FEBS Lett.*, **581**, 2381—2388 (2007).
26) Berrin J. G., McLauchlan W. R., Needs P., Williamson G., Puigserver A., Kroon P. A., Juge N., *Eur. J. Biochem.*, **269**, 249—258 (2002).
27) Day A. J., Canada F. J., Diaz J. C., Kroon P. A., McLauchlan R., Faulds C. B., Plumb G. W., Morgan M. R., Williamson G., *FEBS Lett.*, **468**,

166—170 (2000).
28) Piskula M., Terao J., *J. Nutr.*, **128**, 1172—1178 (1998).
29) Emura K., Yokomizo A., Toyoshi T., Moriwaki M., *J. Nutr. Sci. Vitaminol.*, **53**, 68—74 (2007).
30) Motoyama K., Koyama H., Moriwaki M., Emura K., Okuyama S., Sato E., Inoue M., Shioi A., Nishizawa Y., *Nutrition*, **25**, 421—427 (2008).
31) Hirano T., Kawai M., Arimitsu J., Kuwahara Y., Hagihara K., Shima Y., Narazaki M., Ogata A., Koyanagi M., Kai T., Shimizu R., Moriwaki M., Suzuki Y., Ogino S., Kawase I., Tanaka T., *Allergol. Int.*, **58**, 373—382 (2009).