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# β-Glucosidase Activity in the Rat Small Intestine toward Quercetin Monoglucosides

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**In order to evaluate the positional specificity for a glucoside group in the hydrolysis of flavonoid glucosides in the rat small intestine, β-glucosidase activity was measured with the quercetin monoglucosides, quercetin-3-*O*-β-D-glucopyranoside (Q3G), quercetin-4′-*O*-β-D-glucopyranoside (Q4′G) and quercetin-7-*O*-β-D-glucopyranoside (Q7G), as well as with quercetin-3-*O*-rutinoside (rutin) and *p*-nitrophenyl-β-D-glucopyranoside (NPG) by using the HPLC technique. Enzymes were prepared from rat small intestinal mucosa of the duodenum, jejunum and ileum, among which the enzyme activity of the jejunum was highest for all the glycosides tested. Q4′G was the richest substrate for a β-glucosidase solution among these glycosides, while rutin and NPG were both poor substrates. This suggests that dietary flavonoid glucosides are primarily hydrolyzed and liberated aglycones in the jejunum.**

**Key words:** flavonoid glucosides; quercetin; β-glucosidase

Flavonoids mainly consist of the glycosides of anthocyanidines, flavones, flavonols and flavanones in nature.[1] They are widely distributed in plant foods and in such beverages as tea and wine.[2–4] Epidemiological studies[5–8] have shown that the consumption of green vegetables, green tea, red wine or fresh fruit is effective for reducing the risk of human cancer. It is also associated with lowering the risk of coronary heart disease.[9] Fruits and vegetables are the principal sources of antioxidative ingredients, *i.e.*, vitamins C and E, carotenoids, flavonoids and dietary fibers. As one of the antioxidative nutrients, flavonoids may play an important role in the prevention of these life-style-related diseases.

During the digestion process of foods containing flavonoid glycosides, it appears dietary flavonoid glycosides are mostly hydrolyzed to aglycones by β-glucosidase from the gut flora and finally decomposed to in low-molecular-weight compounds.[10] β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21.) has been isolated from human feces, animal intestines and some bacteria.[11–15] This enzyme catalyzes the hydrolysis of substrate β-glucosidic linkages between glucose and alkyl, aryl or saccharide groups. In hamsters, β-glucosidase is present in the microvillus membrane of the intestinal brush border.[16] Considering flavonoid absorption, it is significant whether flavonoids glycosides are hydrolyzed or not in the small intestine.

Paganga and Rice-Evans[17] have recently reported that dietary flavonoid glycosides were present in the form of quercetin glycosides in human blood plasma. It was indicated that flavonoid glycosides were absorbed into the intestine without hydrolysis. Furthermore, Shimoi *et al.* have succeeded in detecting quercetin and kaempferol in human plasma after the intake of a flavonoid-rich diet (Shimoi *et al.*, personal communication), suggesting that flavonoid glycosides would be hydrolyzed by glycosidase during intestinal absorption.

The main purpose of this study is to investigate substrate differences for rat small intestinal β-glucosidase toward quercetin monoglucosides. The positional specificity of β-glucosidase activity toward groups of flavonoid glycosides is of great interest because dietary flavonoids consist of groups of glycosides that are attached at various positions. We used quercetin monoglucosides, *i.e.*, quercetin-3-*O*-β-D-gluco-pyranoside (Q3G), quercetin-7-*O*-β-D-glucopyranoside (Q7G) and quercetin-4′-*O*-β-D-glucopyranoside (Q4′G), as well as rutin (quercetin-3-*O*-β-rutinoside) for the enzyme assay. In addition, the specificity for *p*-nitrophenyl-β-D-glucopyranoside (NPG) was used as an artificial standard substrate.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) was obtained from Sigma (St. Louis, MO, U.S.A.) and recrystallized from methanol. Q3G, Q7G and Q4′G were chemically synthesized as described previously.[18,19] Rutin was purchased from Funakoshi Co. (Tokyo, Japan), while NPG, 2-mercaptoethanol and phenylmethylsulfonyl fluoride were obtained from Wako Pure Chemicals (Osaka, Japan).

Intestinal tissue samples were prepared from 9-weeks old male Sprague-Dawley rats that had been fed on laboratory feed *ad libitum* and were not fasted before the experiment. After the rats had been killed by carotid artery cleavage, their small intestines were rinsed with

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*Abbreviations*: Q3G, quercetin-3-*O*-β-D-glucopyranoside; Q4′G, quercetin-4′-*O*-β-D-glucopyranoside; Q7G, quercetin-7-*O*-β-D-glucopyranoside; KRP buffer, Krebs-Ringer phosphate buffer; NPG, *p*-nitrophenyl-β-D-glucopyranoside.

an ice-cooled Krebs-Ringer Phosphate (KRP) buffer (pH 7.4). After being dried on a filter paper (No. 2), the first 20-cm segment distal to the pylorus on the filter paper was removed and is designated as the duodenum. The next 20-cm segment between the pylorus and the caecume was removed and is designated as the jejunum. The 3rd portion, designated as the ileum, was the distal 20-cm segment of the intestine proximal to the caecume. Each segment was opened and rubbed with a micro spatula to obtain intestinal mucosa, and all intestinal mucosa was stored at −40°C until needed for use. The supernatant from the rat intestinal homogenate by the method of Nakano and Gregory[20] and used it for the β-glucosidase assay. Protein was determined by the Bradford method, using γ-globulins derived from bovine plasma as a standard.[21]

All substrates were dissolved in 5 μl of dimethyl-sulfoxide and added to 465 μl of a 10 mM sodium phosphate buffer, which was adjusted to the optimum pH 6.0 of rat small intestinal β-glucosidase.[22] After preincubating for 3 min at 37°C, the reaction was initiated by adding 30 μl of an enzyme solution to a KPR buffer (pH 6.0) containing a substrate. The final concentration of each substrate was 100 μM in the reaction mixture. The reaction mixture was incubated for 20 min at 37°C while continuously shaking, and was terminated by mixing with 750 μl of methanol. After centrifugating at 13,000×*g* for 10 min at 4°C, the supernatant was passed through a membrane filter (DISMIC filter, cellulose acetate, 0.45 μm pore size, Advantec, Tokyo, Japan) and subjected to an HPLC analysis.

Quercetin liberated from the glucosides was measured by HPLC in a reversed-phase column (TSK-gel ODS-80Ts, I.D. 4.6×150 mm, 5 μm particle size, Tosoh Co., Tokyo, Japan) with the same guard column. The eluting solvent was a mixture of methanol, distilled water and 90% formic acid (49:50:1, v/v). Distilled water was used after being passed through a membrane filter (cellulose acetate, 0.2 μm pore size, Advantec). The flow rate was 1.0 ml/min, and the eluate was monitored by its absorption at 370 nm. To identify *p*-nitrophenol as an NPG aglycone, the eluting solvent was 70% aqueous methanol and was detected at 320 nm. The analytical column and flow rate were the same as those used in the analysis for quercetin.

The HPLC apparatus consisted of a pump (Gulliver PU-980), detector (Gulliver UV-970 or Hitachi 655 UV monitor), and data processor (Gulliver 807-IT integrator). All of these components are products of JASCO Corporation (Tokyo, Japan) except for the 655 UV monitor (Hitachi, Tokyo, Japan). Enzyme activity is defined as moles of quercetin generated from the reaction mixture per hour per mg of protein. Statistical analyse were carried out by one-way analysis of variance (ANOVA).

Typical HPLC chromatograms of the reaction mixtures after the hydrolysis of quercetin glucosides and NPG are shown in Fig. 1. Quercetin and NPG could be liberated from their glycosides by the reaction with the enzyme (Figs. 1A and 1B). Neither peak was detected without the addition of an enzyme solution to the mixture (data not shown). It was found that the β-glucosidase inhibitor, castanospelmine, completely inhibited the hydrolysis of Q4′G (data not shown).

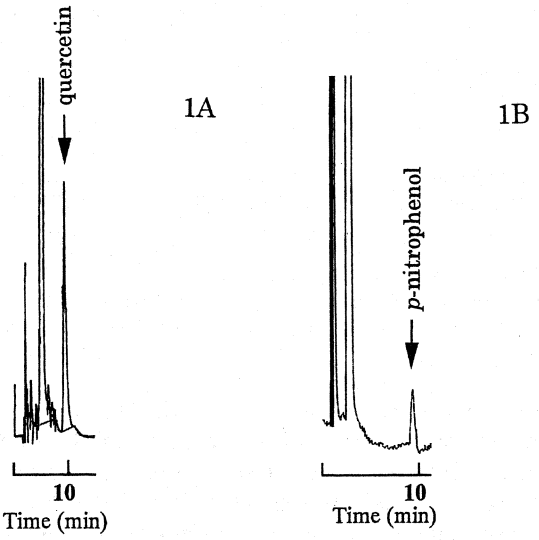


**Fig. 1.** Reversed-phase HPLC of the Reaction Mixture for the Hydrolysis of Q4′G (A) and NPG (B).

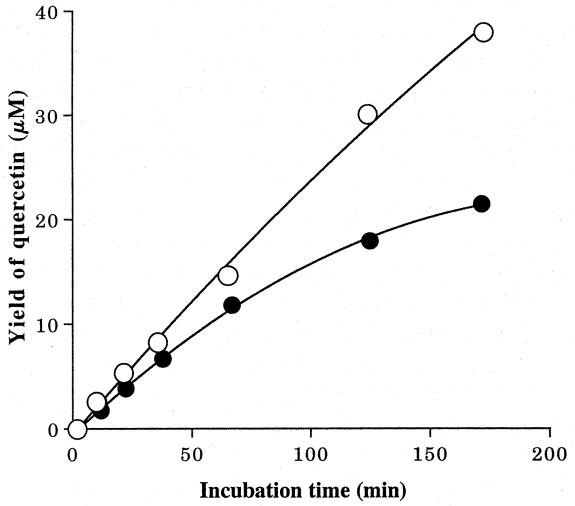


**Fig. 2.** Time-course Plots of the Hydrolysis of Q4′G in the Jejunum Preparation.

The assay mixture consisted of a 91 μM (○) or 46 μM (●) substrate and 30 μl of a β-glucosidase solution in a 10 mM phosphate buffer (containing 10 mM 2-mercaptoethanol and 1 mM phenylmethyl surfonyl fluoride) at pH 6.0.

Figure 2 shows time-course plots of the hydrolysis of Q4′G at 91 μM and 46 μM. The extract as the enzyme source was prepared from the jejunum. It was found that Q4′G underwent hydrolysis linearly for 60 min, and then the reaction underwent became saturated until 170 min. Therefore, in the subsequent experiment, we compared the rate of quercetin liberation from various substrates during the first 20 min.

Table 1 shows β-glucosidase activities in the three small intestinal segments with quercetin glycosides. While β-glucosidase activity was detected in all segments

**Table 1.** β-Glucosidase Activities in the Three Small Intestinal Segments with Quercetin Glycosides

| Intestinal portion | Substrate | | | | |
|---|---|---|---|---|---|
| | Rutin | Q3G | Q4′G | Q7G | NPG |
| | $\times 10^{-9}$ mol/hr/mg protein | | | | |
| Duodenum | $2.84 \pm 0.07^{a,A}$ | $4.98 \pm 1.50^{a,A}$ | $10.01 \pm 2.37^{b,A}$ | $3.89 \pm 0.47^{a,A}$ | $3.45 \pm 1.54^{a}$ |
| Jejunum | $10.22 \pm 2.06^{a,B}$ | $28.77 \pm 5.94^{B}$ | $59.17 \pm 15.14^{B}$ | $25.77 \pm 6.05^{B}$ | $3.52 \pm 0.94^{a}$ |
| Ileum | $6.14 \pm 1.72^{a,c}$ | $11.22 \pm 0.87^{a,d,C}$ | $20.00 \pm 2.09^{b}$ | $10.53 \pm 1.44^{a,C}$ | $2.23 \pm 0.41^{a}$ |

Each value is the mean ± SE (n=3).

The reaction mixture consisted of 100 μM of a substrate and 30 μl of an enzyme solution in the buffer shown in the legend to Fig. 2. Different superscript letters, a–d (comparing among substrates), and A–C (comparing among intestinal portions), indicate significant differences ($P<0.05$).

of the small intestine, it appeared that the jejunum was more active than the other segments in the quercetin glucoside hydrolytic reaction of three quercetin glucosides ($P<0.05$). This indicates that the hydrolysis of flavonoid glucosides occured mainly in the jejunum.

Among the quercertin monoglucosides, Q4′G was hydrolyzed faster than Q7G and Q3G. The activity of the jejunum toward Q4′G was about twice as high as it was toward Q7G and Q3G. The β-glucosidase activities for rutin and NPG were lower than those of such monoglucosides in each segment. A statistical comparison of enzyme activities toward three quercetin monoglucosides in the jejunum shows that there was no significant difference. Rutin seems to have been a poor substrate because the linkage of rutinose is not suitable for this β-glucosidase as has previously been indicated.[13] Amygdalin was also hydrolyzed in the jejunum,[24] suggesting that low-molecular-weight glycoside compounds were enzymatically hydrolyzed there.

Freer[11] and Tamura *et al.*[12] have shown that β-glucosidase purified from fecal flora catalyzed the liberation of glycoside groups from rutin and several other glycosides. With flavonoid hydrolysis in the intestines, almost all reports have used the enzyme extracted by intestinal microflora in faces. It has been suggested that flavonoids were decomposed by gut flora in the large intestine. β-Glucosidase activity in the small intestine has been studied for the metabolism of pyridoxine (vitamin B6)[20,22] and amygdalin.[23,24] In this present study, it was obvious that flavonoid glucosides were hydrolyzed in the same way as other natural glucosides and that β-glucosidase had substrate specificity to flavonoid glucoside isomers.

Patchett *et al.*[13] have described how the hydrolysis rate of an aryl β-linkage glucoside is independent of the electron-withdrawing power of the aryl group with respect to the developing phenoxide anion during the first step of catalysis. We have already indicated that the hydroxyl group at the C-4′ position of quercetin played an essential role in radical scavenging that was different from the hydroxyl groups at positions C-7 and C-3.[25] Therefore, the position with the most liability toward β-glucosidase coincides with the position responsible for exerting the antioxidative action. In contrast, the hydrolysis by β-glucosidase of rutin was lower than that of Q3G, although both compounds contained a glycoside group at the C-3 position and Q3G could act as the substrate for the β-glucosidase activity of the intestines. Some reports have indicated that rutin was metabolized by large intestinal microflora.[10,26]

In conclusion, the hydrolysis of quercetin glucosides in intestinal tissue may be involved in enhancing of the antioxidative activity of quercetin *in vivo* by releasing aglycones during intestinal absorption.

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