Flavonoid glucuronides are substrates for human liver β-glucuronidase

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Abstract Quercetin glucuronides are the main circulating metabolites of quercetin in humans. We hypothesise that the potential availability of the aglycone within tissues depends on the substrate specificity of the deconjugating enzyme β-glucuronidase towards circulating flavonoid glucuronides. Human tissues (small intestine, liver and neutrophils) exhibited β-glucuronidase against quercetin glucuronides. The various quercetin glucuronides were deconjugated at similar rates, but liver cell-free extracts were the most efficient and the activity was completely inhibited by saccharo-1,4-lactone (a β-glucuronidase inhibitor). Furthermore, pure recombinant human β-glucuronidase hydrolysed various flavonoid glucuronides, with a 20-fold variation in catalytic efficiency ($k_{cat}/K_m = 1.3\times10^3$ M^{-1} s^{-1} for equol-7-*O*-glucuronide and 26×10^3 M^{-1} s^{-1} for kaempferol-3-*O*-glucuronide). Similar catalytic efficiencies were obtained for quercetin *O*-glucuronides substituted at different positions. These results show that flavonoid glucuronides can be deconjugated by microsomal β-glucuronidase from various human cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Human; β-Glucuronidase; Flavonoid; Glucuronide; Quercetin; Turnover

1. Introduction

The flavonoids represent the most common and widely distributed group of plant polyphenolic compounds. Quercetin, a major flavonol, has free radical scavenging activity [1] and many biological activities such as antioxidant, antithrombotic and anti-carcinogenic activities [2]. Some epidemiological studies have suggested that a high flavonol intake is associated with a reduced incidence of cardiovascular disease [3,4].

The small intestine, as the first site of exposure to xenobiotics, is an important site of metabolism of flavonoids. Conjugation of the flavonoids in the small intestine and liver to glucuronides, sulphates or methylated derivatives has been shown [5–7], although the position of conjugation for many is not known. Recently Day et al. showed that quercetin was conjugated by uridine-diphosphate-glucuronosyltransferase (UGT) from human liver extracts at four different hydroxyl groups, but these conjugates had vastly different abilities to inhibit xanthine oxidase and lipoxygenase in vitro [8].

Conjugation is a common detoxification reaction leading to increased solubility of compounds, which is important for excretion. Flavonoid glucuronides are likely to be excreted in the bile due to their increased molecular weight. The flavonoids can then undergo enterohepatic circulation as bacterial β-glucuronidases hydrolyse the glucuronide enabling reabsorption of the free aglycone. The liver, however, may also play a role in the turnover of flavonoid glucuronides in vivo due to the expression of β-glucuronidase in addition to UDP-glucuronosyltransferase.

Human β-glucuronidase (EC 3.2.1.31) is an acid hydrolase enzyme expressed in many tissues and body fluids [9]. There is large interindividual variation in its activity and expression in the liver, kidney and serum [10]. One third of the liver β-glucuronidase is located in the lumen of the endoplasmic reticulum (ER) [11] and has been suggested to play a role in hepatic cycling [12]. β-Glucuronidase is also found in the lysosomal fraction of the cell [11] and has a role in hydrolysis of steroid hormone glucuronides [13]. Thus, the activity of β-glucuronidase may modify the net rate of glucuronide formation and may influence the pathway of metabolism by directing conjugation at specific positions. The aim of this paper was to investigate the level of β-glucuronidase activity from various human sources towards quercetin glucuronides and determine the affinity of recombinant human β-glucuronidase for flavonoid glucuronides conjugated at various positions.

2. Materials and methods

2.1. Materials

All material and chemical reagents were obtained from Sigma (Poole, UK) unless otherwise stated and were analytical or HPLC grade where applicable. Water was purified via a Millex Q plus system (Millipore, Watford, UK). Quercetin-3-*O*-glucuronide was purified from green bean tissue [14] and kaempferol-3-*O*-glucuronide was isolated from endive [15]. Daidzein-7-*O*-glucuronide was chemically synthesised by Needs et al. [16]. MS and NMR have previously confirmed the identity of these compounds. All flavonoids were checked for purity prior to use by HPLC and were >98% pure. All substrates were stored in aqueous methanol and were stable in this form at −20°C for over 6 months. Prior to an experiment, an aliquot of the flavonoid glucuronide stock was diluted in 20 mM sodium phosphate buffer pH 7.2. The final concentration of the methanol was less than 0.3% (v/v) in the reaction mixture, which was found not to interfere with the assay.

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Abbreviations: UGT, uridine-diphosphate-glucuronosyltransferase; *p*-NPGlcA, *p*-nitrophenol glucuronide; UDPGA, uridine-diphosphate-glucuronic acid; ER, endoplasmic reticulum

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2.2. Preparation of equol-7-, quercetin-4′- and quercetin-7-O-glucuronides

General method: Solvents were dried over 3 Å molecular sieves; solids in vacuo over P_2O_5. Evaporations were performed in vacuo at 50°C. TLC used silica 60/UV254 plates (Macherey–Nagel) eluted with 5% MeOH/CH_2Cl_2. Analytical HPLC, and HPLC-ESMS, using a 5 μm Prodigy ODS3 column (250×4.4 mm) eluted at 1 ml min^{-1} with UV detection (205, 280 nm): eluant A 80% for 5 min (isocratic); to 10% A at 35 min (gradient). Preparative HPLC using a 5 μm Prodigy ODS3 column (Phenomenex Inc., 250×21.2 mm+60×21.2 mm guard) eluted at 5 ml min^{-1}. Eluant A – 0.1% CF_3COOH (TFA); eluant B – CH_3CN. 80% A for 15 min (isocratic); then to 10% A at 75 min (gradient). NMR spectra were run at 27°C on a JEOL JNM-EX270 spectrometer using the residual solvent's absorption for calibration. Electrospray mass spectroscopy (ESMS), and HPLC-ESMS analyses were performed on a Micromass Quattro II mass spectrometer (Manchester, UK).

2.3. Quercetin-7-O-glucuronide and quercetin-4′-O-glucuronide

3′,4′,4,5-Tetrabenzoylquercetin (300 mg, 420 μmol) [17], 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (185 mg, 1.1 eq), Ag_2CO_3 (127 mg, 1.1 eq), 3 Å sieves (500 mg) and dry CH_2Cl_2 (5 ml) were stirred, in the dark, under Ar (5 min). Collidine (60 μl, 1.1 eq) was added. After 3 days, the mixture was filtered (celite). The filtrate and celite rinses (5% MeOH/CH_2Cl_2, CH_2Cl_2 2×50 ml) were combined and washed with 1 M HCl (50 ml), H_2O (50 ml), 0.1 M $Na_2S_2O_3$ (50 ml), H_2O (50 ml), saturated $NaHCO_3$ (50 ml), and H_2O (50 ml), and dried ($MgSO_4$). The evaporated solid was suspended in 1 M NaOH (20 ml), stirred under Ar (0°, 60 min), warmed to room temperature (rt), left for 1 h, and heated at reflux (10 min). After cooling Dowex 50W resin (H^+ form, 40 ml) was added, and stirred (10 min); the mixture was filtered, and filtrate and resin washes (50% aq MeOH, 50 ml, MeOH 50 ml) were combined and evaporated. The resultant solid was dissolved in 5% aq MeOH (100 ml), extracted with CH_2Cl_2 (3×75 ml); the aqueous phase was evaporated. The residue was dissolved in 50% aq MeOH (8 ml), and filtered. The products were isolated by preparative HPLC (2×4 ml injections) and were pure by analytical HPLC. Quercetin-7-*O*-glucuronide. Yield 17 mg, 8%. ^1H NMR (CD_3OD): δ 7.76 (d, 1 H, $J_{2',6'}$ 2.0 Hz, H-2′), 7.66 (dd, 1 H, $J_{6',5'}$ 7.6 Hz, H-6′), 6.89 (d, 1 H, H-5′), 6.74 (d, 1 H, $J_{8,6}$ 2.0 Hz, H-8), 6.46 (d, 1 H, H-6), 5.14 (d, 1 H, $J_{1'',2''}$ 7.2 Hz, H-1″), 4.08 (d, 1 H, $J_{5,4}$ 9.2 Hz, H-5″), 3.4–3.7 (m, 3 H, H-2″, H-3″, H-4″). ESMS: *m/z* 479 $[M+H]^+$; 501 $[M+Na]^+$; 477 $[M-H]^-$; 591 $[M+TFA-H]^-$. Quercetin-4′-glucuronide. ESMS: *m/z* 479 $[M+H]^+$; 501 $[M+Na]^+$; 477 $[M-H]^-$; 591 $[M+TFA-H]^-$.

2.4. Equol-7-O-glucuronic acid

Daidzein-7-*O*-glucuronic acid (20 mg, 46 μmol) was methyl esterified by stirring in MeOH with Dowex 50W (H^+) (3 days, rt). After filtration and evaporation, the residue, dissolved in EtOH (95%, 5 ml), was added to Pd/C (10%, 50 mg). The mixture was stirred under H_2 for 30 h, and filtered (celite). The filtrate and celite washes (MeOH, 20 ml, and 50% CH_2Cl_2/MeOH, 20 ml) were combined and evaporated. After deesterification with Na_2CO_3 [16] the product was purified by preparative HPLC. Yield 3 mg (15%). ^1H NMR (CD_3OD): δ 7.10 (d, 2 H, $J_{2',3'}$ 8.6 Hz, H-2′, H-6′), 6.99 (d, 1 H, $J_{5,6}$ 8.2 Hz, H-5), 6.76 (d, 2 H, H-3′, H-5′), 6.60 (dd, 1 H, $J_{6,8}$ 2.4 Hz, H-6), 6.53 (d, 1 H, H-8), 4.9 (d, 1 H, partially obscured by HOD peak, H-1″), 4.23 (m, 1 H, H-2a), 3.90–3.95 (m, 2 H, H-2β, H-5″), 3.4–3.7 (m, 3 H, H-2″, H-3″, H-4″), 3.0–3.1 (m, 1 H, H-3), 2.85–2.9 (m, 2 H, 2×H-4). ESMS: *m/z* 417 $[M-H]^-$.

2.5. Enzymatic synthesis of flavonoid glucuronides

Mixed quercetin glucuronides were produced enzymatically from pig liver. Quercetin (200 μM) was incubated for 180 min at 37°C in HEPES buffer (25 mM, pH 7.2 containing 10 mM $MgCl_2$), a post-lysosomal fraction of pig liver, UDP-glucuronic acid (final concentration, 4 mM) and UDP-glucosamine (final concentration, 2 mM). The extent of conjugation rate was greater than 95%.

2.6. Preparation of cell-free extracts

Samples of human liver were obtained from redundant tissues of surgical specimens from patients undergoing hepatic surgery. Samples of small intestine were purchased from Anatomic Gift Foundation (Laurel, MD, USA). Human blood was obtained from healthy volunteers. These studies were approved by the Norwich District Ethics committee and were carried out in accordance with the Declaration of Helsinki of the World Medical Association. All patients gave informed consent to the work. A similar version of the previously published method of Day et al. [18] to prepare cell-free extracts of human liver and small intestine was adopted. Blood neutrophils were isolated according to the method of Marshall et al. [19].

2.7. Assays for β-glucuronidase activity

All assays were performed at pH 7.2. *p*-Nitrophenol glucuronide (*p*-NPGlcA, 10 mM) was incubated with cell-free extracts of human liver, human small intestine, human faecal samples and human blood neutrophils and sodium phosphate buffer (20 mM, pH 7.2) in a final volume of 0.1 ml. All samples were incubated at 37°C for 30 min, during which time linear rates were obtained. Reactions were quenched by addition of 0.9 ml 100 mM sodium borate, pH 9.3. β-Glucuronidase activity was determined by release of *p*-nitrophenol at 400 nm ($E = 18\,300$ M^{-1} cm^{-1}). Protein content of the human tissues was measured using the method of Lowry et al. [20], using bovine serum albumin as a standard. Saccharo-1,4-lactone (1 mM) was added to some assays as indicated in the legends. A quercetin glucuronide mixture (100 μM) was tested as a substrate under the conditions described above. Incubations were for 180 min and were analysed by HPLC after extraction into methanol. Human tissue samples were obtained from at least three individuals and experiments were carried out in triplicate, with nine observations in total for each individual. Recombinant human β-glucuronidase was prepared as described previously by Oshima et al. [21]. To determine the K_m and V_{max}, substrate was incubated with pure human recombinant β-glucuronidase (final concentration 1.1 μg ml^{-1}) and sodium phosphate buffer in a final volume of 0.2 ml. Reactions were incubated at 37°C for 30 min during which time the reaction rate was linear. Reactions were stopped by addition of 200 μl of methanol containing 1 mM ascorbic acid, followed by centrifugation at 13 500×*g* for 10 min at 4°C. The supernatant was filtered through 0.22 μm PTFE filters (Chromos Express, Macclesfield, UK) and analysed by HPLC. The K_m and V_{max} were calculated for the various substrates using Graffit software. The data were fitted to Michaelis–Menten equations and results were based on the conversion of flavonoid glucuronides to their respective aglycones. One unit of activity is defined as the amount of enzyme releasing 1 nmol of product per min at 37°C, pH 7.2.

2.8. HPLC analysis

A modified version of the previously published analytical HPLC method of Price et al. [22] was adopted. An external standard of rutin was run approximately every six runs.

Table 1
Deconjugation of quercetin glucuronides by cell-free extracts of human small intestine, liver, neutrophils and faeces

	β-Glucuronidase activity towards p-NPGlcA (nmol mg^{-1} protein min^{-1})	Quercetin glucuronide deconjugation (%)
Human small intestine	3.6 ± 0.2	57 ± 14
Human liver	2.8 ± 0.3	100 ± 10
Human liver+saccharo-1,4-lactone	n.d.	0 ± 2
Human neutrophils	1.5 ± 0.3	68 ± 8
Human faeces	4.0 ± 0.3	60 ± 8

Mean ± S.E.M. $N = 3$ independent experiments from samples from at least three individuals, with a total of nine observations. n.d., not determined.

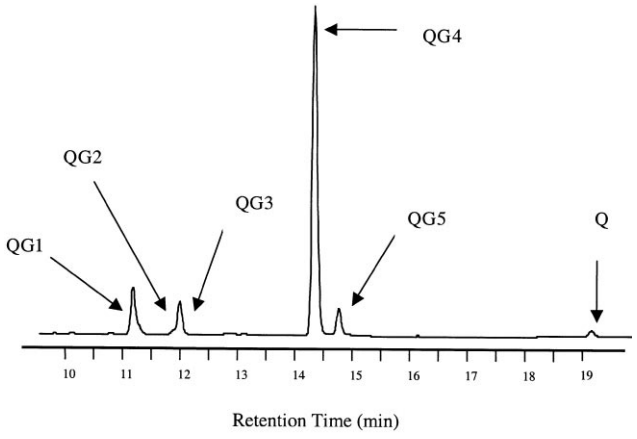

Fig. 1. HPLC chromatogram of quercetin glucuronides. QG1 (*m/z* 654, *m/z* 303) is a di-glucuronide, QG2 (*m/z* 479, *m/z* 303) co-eluted with quercetin-3-glucuronide, QG3 with quercetin-7-*O*-glucuronide, QG4 with quercetin-4′-*O*-glucuronide and QG5 with quercetin-3′-*O*-glucuronide standards. Similarly, Q (*m/z* 303) co-eluted with a quercetin standard. The molar percentages of each mono-*O*-glucuronide within the mixture are: 3-*O*-glucuronide-7-*O*-glucuronide, 6.5%; 4′-*O*-glucuronide, 85%; 3′-*O*-glucuronide, 6.5%; free quercetin, 2%.

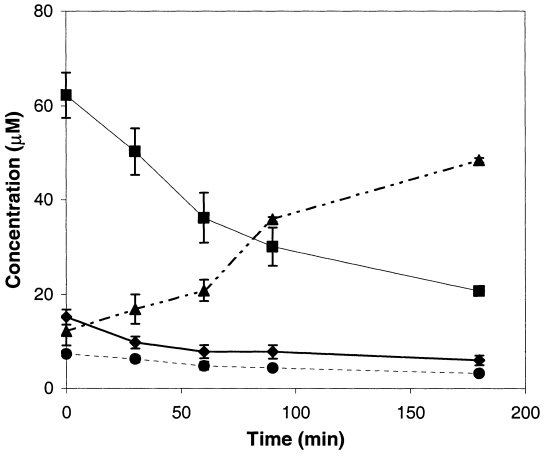

Fig. 2. Deconjugation of quercetin glucuronides by human blood neutrophils. Total mixed quercetin glucuronides (100 μM) were incubated for 180 min with cell-free extracts of human neutrophils. (▲) quercetin; (■) quercetin-4′-*O*-glucuronide; (♦) quercetin-3/7-*O*-glucuronide and (●) quercetin-3′-*O*-glucuronide. Errors bars represent standard error of the mean, $N = 3$ independent experiments with samples obtained from at least three individuals, total of nine observations for each individual.

3. Results

Five quercetin glucuronides were produced from an incubation of quercetin with a cell-free extract of pig liver (Fig. 1). Co-elution with known quercetin glucuronide standards allowed the *O*-conjugation position to be determined for QG2, QG3, QG4, QG5 (3-, 7-, 4′-, 3′-), respectively. LC-MS confirmed QG1 to be a quercetin di-glucuronide, although the positions of conjugation were not further elucidated.

All the human tissues investigated exhibited β-glucuronidase activity towards *p*-NPGlcA (Table 1). Incubation of a mixture of quercetin glucuronides with the cell-free extracts (adjusted to have equivalent *p*-NPGlcA activity) demonstrated the ability of these tissues to deconjugate quercetin glucuronides (Table 1). The activity of the most active sample, liver cell-free extract, was inhibited completely by saccharo-1,4-lactone, a specific β-glucuronidase inhibitor, demonstrating that all of the activity was due to β-glucuronidase. Although the faecal β-glucuronidase was more active than the human tissues towards *p*-NPGlcA, quercetin glucuronides were hydrolysed more rapidly by human liver cell-free extracts, presumably owing to the different specificities of the colon microflora enzymes compared to the human endogenous β-glucuronidase. Fig. 2 shows the hydrolysis of the individual quercetin glucuronides to quercetin by cell-free extracts of human blood neutrophils. Similar profiles were seen for the other tissues, illustrating that the rate of deconjugation does not appear to be affected by the position of glucuronide conjugation. The di-glucuronide of quercetin was also hydrolysed at a similar rate to the mono-glucuronides.

To further demonstrate that the activity in the cell-free extracts was due to β-glucuronidase, the ability of pure recombinant human β-glucuronidase to hydrolyse various (iso)flavonoid glucuronides conjugated in various positions was assessed. β-Glucuronidase hydrolysed both the flavonol and isoflavone substrates, demonstrating lower K_m values and higher catalytic activity constants than that obtained for the artificial substrate, *p*-NPGlcA (Table 2). The K_m values for all the substrates showed a 10-fold range. However, the K_m of *p*-NPGlcA was much higher, indicating a much poorer apparent affinity. The catalytic efficiency of the enzyme (k_{cat}/K_m) showed a 20-fold difference in activity. β-Glucuronidase was active on isoflavonoid glucuronides, and the position of conjugation did not influence this activity. The catalytic activity constants for quercetin-4′-*O*-glucuronide, quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide were similar, indicating that the position of conjugation does not affect the activity of β-glucuronidase.

4. Discussion

Glucuronides of flavonoids have been observed in the plasma of humans and rats [5,23–25]. Conjugation with glucu-

Table 2
Kinetic constants for the hydrolysis of flavonol and isoflavone glucuronides by pure human recombinant β-glucuronidase

Substrate	K_m (μM)	V_{max} (U mg^{-1} β-glucuronidase)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Kaempferol-3-*O*-glucuronide	25 ± 4	1179 ± 68	0.65	26.0×10^3
Quercetin-7-*O*-glucuronide	237 ± 30	2937 ± 163	1.65	6.9×10^3
Quercetin-3-*O*-glucuronide	167 ± 15	3448 ± 106	1.90	11.4×10^3
Quercetin-4′-*O*-glucuronide	48 ± 11	465 ± 45	0.26	5.4×10^3
Daizein-7-*O*-glucuronide	272 ± 30	1942 ± 82	1.07	3.9×10^3
Equol-7-*O*-glucuronide	266 ± 33	626 ± 32	0.35	1.3×10^3
p-NPGlcA	1400 ± 415	1221 ± 140	0.67	47

ronic acid is mediated by UGTs, which are predominantly located in the ER [26] and in the nuclear envelope of hepatocytes and cells in other organs. Microsomal β-glucuronidase is located in the lumen of the ER where it is stabilised in a complex with egasyn [27]. The presence of both UGT and β-glucuronidase in close subcellular proximity within the cell may facilitate deconjugation and therefore regulate the rate of net glucuronidation, depending on the specificity of each enzyme. Both UGT and β-glucuronidase are active at physiological pH in rat liver microsomes [28], but the optimum pH of β-glucuronidase is between 4.5–6.0. Day et al. [8] determined the K_m of UGT specificity towards flavonoid aglycones in human cell-free extracts to be ~ 5 μM. This K_m is between 10–50-fold lower than the K_m we obtained for β-glucuronidase activity towards (iso)flavonoid glucuronides. In case the two enzymes are located in the same compartment, this would suggest that the pathway would favour the formation of glucuronides rather than their hydrolysis.

Although the liver is considered to be the main drug-metabolising organ in the body, the intestinal mucosa often represents the first exposure site to compounds and may play a role in first-pass metabolism of xenobiotics. Rat and human intestinal UGT glucuronidate flavonoids (e.g. [6]). Formation of glucuronide conjugates after passage across the small intestine indicates that the form reaching and entering the hepatocyte is predominantly a glucuronide derivative. We have demonstrated the ability of human β-glucuronidase to deconjugate flavonoid glucuronides. However, for this process to occur, the glucuronide must first enter the cell and then reach the same compartment as the β-glucuronidase enzyme. This is conceivable, since other conjugates, such as glucuronides of bilirubin or estradiol, can be actively transported into the liver by OATP2 [29], and paracetamol glucuronide enters cells via a carrier mediated transporter [30].

Human eosinophils and neutrophils contain lysosomal enzymes that are released after cell activation. We have shown β-glucuronidase activity in neutrophils after releasing the cell contents and in agreement with Marshall et al. [19]. Furthermore, Shimoi et al. [31] demonstrated the release of lysosomal β-glucuronidase from neutrophils following stimulation to induce inflammation in the cells, resulting in deconjugation of flavone luteolin-7-*O*-glucuronide to luteolin. These results suggest that lysosomal β-glucuronidase released from neutrophils can deconjugate flavonoid glucuronides to the free aglycone, when injury such as inflammation occurs.

Mammalian microsomal and lysosomal β-glucuronidase are derived from the same gene. They are catalytically and immunologically identical but differ in their degree of glycosylation. The physiological role of microsomal β-glucuronidase remains to be elucidated. However, Whiting et al. [20] suggested a role for microsomal β-glucuronidase in the deconjugation of bilirubin in rats. It has also been shown that microsomal β-glucuronidase activity may be stimulated and net hepatic glucuronide production decreased when isolated perfused liver receptors are activated by α-adrenergic compounds [32]. We have shown that β-glucuronidase in human tissues could play a role in the turnover of flavonoids, provided that the flavonoid conjugate reaches the same cellular compartment.

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