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Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans

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Summary *Background* Pharmacokinetic studies have shown that the small intestine is the major site of absorption for many flavonoid glucosides. Flavonoids are generally present as glycosylated forms in plants and foods, but there is increasing evidence that the forms reaching the systemic circulation are glucuronidated, sulphated and methylated derivatives. Hence, first-pass metabolism (small intestine-liver) appears to involve a critical deglycosylation step for which the mechanisms are not known. *Aims* To explore the hypothesis that deglycosylation is a prerequisite to absorption and metabolism of dietary flavonoid glycosides, to identify the enzymes responsible, and relate their specificities with absorption kinetics. *Methods* Flavonoid glycoside hydrolysing enzymes were isolated from samples of human small intestine and liver using chromatographic techniques. The proteins were characterised with respect to the cellular fraction with which they were associated, molecular weight, specificity for various substrates, and cross-reactions with antibodies. Cellular models were used to mimic the small intestine. *Results* Protein extracts from human jejunal mucosa were highly efficient in hydrolysing flavonoid glycosides, consistent with an enterocyte-mediated deglycosylation process. Considerable inter-individual variation was observed [e. g. range, mean and standard deviation for rate of hydrolysis of quercetin-3-glucoside (n = 10) were 6.7–456, 96, and 134 nmol min^{-1} (mg protein)$^{-1}$, respectively]. Two β-glucosidases with activity towards flavonoid glycosides were isolated from human small intestine mucosa: lactase-phlorizin hydrolase (LPH; localised to the apical membrane of small intestinal epithelial cells) and cytosolic β-glucosidase (CBG), indicating a role of human LPH and CBG from small intestine in flavonoid absorption and metabolism. Hydrolysis of flavonoid glycosides was only detected in cultured cells exhibiting β-glucosidase activity. *Conclusions* The absorption of dietary flavonoid glycosides in humans involves a critical deglycosylation step that is mediated by epithelial β-glucosidases (LPH and CBG). The significant variation in β-glucosidase activity between individuals may be a factor determining variation in flavonoid bioavailability.

Key words dietary antioxidants – lactase phlorizin hydrolase – cytosolic beta-glucosidase – flavonols – isoflavones – anthocyanins

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Abbreviations

BSA	bovine serum albumin
CBG	cytosolic β-glucosidase
DTT	dithiothreitol
DMEM	Dulbecco's Modified Eagle Media
DMSO	dimethylsulphoxide
EDTA	ethylene diamine tetraacetic acid
HPLC	high pressure liquid chromatography
HPLC-DAD	HPLC with on-line diode array detection
LPH	lactase-phlorizin hydrolase
MDCK	Madin-Darby canine kidney
MDCK-LPH	MDCK cells expressing full-length human *LCT cDNA*
4NP	4-nitrophenol
MRP	multi-drug resistance protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pNPGlc	4-nitrophenyl-β-D-glucopyranoside
PP	polyphenol
Q3Glc	quercetin-3-glucoside (isoquercitrin)
Q4'Glc	quercetin-4'-glucoside (spiraeoside)
SDS	sodium dodecyl sulfate
SGLT-1	sodium-dependent glucose transporter-1
TEER	transepithelial electrical resistance

Introduction

Flavonoids and isoflavones have attracted considerable interest from both food producers and consumers due to their antioxidative activities and apparent health-promoting properties. Epidemiological data have consistently shown an inverse relationship between the consumption of fruits and vegetables and the incidence of disease [1]. (Iso)flavonoids are potent antioxidants *in vitro*, and it has become a popular belief that these polyphenols, as part of dietary fruits and vegetables, provide protection against disease by functioning as antioxidants *in vivo* [2]. Epidemiological studies have suggested associations between the consumption of (iso)flavonoids and (iso)flavonoid-rich foods and the incidence of coronary heart disease [3–5], various cancers [6, 7], stroke [8] and osteoporosis [9]. However, it is noteworthy that in some studies the correlation between consumption of flavonoids and the risk of disease was weak, not reaching statistical significance [3, 10].

Flavonoids are powerful antioxidants *in vitro* [11] and have been shown to be effective anti-carcinogenic and anti-atherogenic agents in various animal and cell models [12]. However it is not known if these anti-carcinogenic properties are directly and/or entirely due to their antioxidant properties (see [12]). Indeed, there is an abundance of indirect evidences that various flavonoids protect against damage to cellular macromolecules (lipids, DNA, proteins) that support a role in prevention of diseases where oxidative stress is an important component of the disease process [13]. However, direct evidence for this mechanism has proved elusive and there is a clear discrepancy between the reported potency of flavonoids *in vitro* and their role *in vivo* as measured using epidemiological and human intervention studies. More recently, the biological activity of (iso)flavonoids has been assessed through their ability to affect cellular function by mediating gene expression

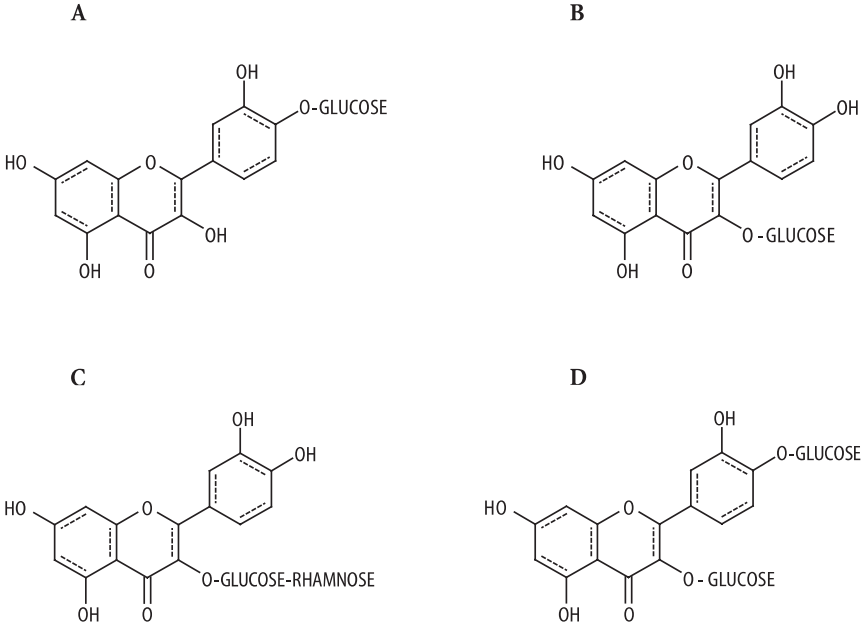

Fig. 1 Structure of the poly-hydroxylated flavonoid quercetin showing examples of several common glycosylated derivatives that are present in plants and foods. **A** Quercetin 4′-glucoside (spiraeoside); **B** quercetin 3-glucoside (isoquercitrin); **C** quercetin 3-rutinoside (rutin); **D** quercetin 3,4′-diglucoside

and signal transduction [14, 15] rather than through a direct antioxidant effect. In order to bridge this gap, it is important to understand the absorption and metabolism processes of dietary flavonoids, and to identify the resulting metabolites *in vivo*.

Flavonoids are present in plants almost exclusively as β-glycosides (Fig. 1). Furthermore, most industrial and domestic food processing procedures do not lead to cleavage of the glycosidic linkage [16–19] and hence flavonoids in foods are generally present as glycosides. Notable exceptions are fermented soy-based products such as tempeh and ang sak which are exposed to microbial β-glucosidases which leads to the release of isoflavone aglycones including genistein and daidzein from their parent compounds (genistin and daidzin) [20]. The position, nature and amount of glycosylation depend on the plant species and is often a useful discriminatory tool used in plant identification and food authenticity tests.

Several studies have shown that flavonoids including quercetin reach plasma following ingestion of flavonoid supplements or flavonoid-rich foods [21–24]. However, polyphenol glycosides are relatively hydrophilic and do not diffuse passively across biological membranes. (Iso)flavonoid aglycones may be absorbed from the stomach while their respective glucosides are not [25, 26]. Until recently, it was commonly accepted that polyphenol glycosides remained intact until they reached the colon where they underwent deglycosylation mediated by microbial enzymes. However, intervention studies indicated that the primary site of absorption of some flavonoid glycosides was the small intestine [21, 27–29]. This demonstrated that quercetin glucosides were absorbed earlier, resulted in a higher plasma peak concentration of total quercetin, and were more bioavailable than quercetin aglycone or quercetin rutinoside (rutin, quercetin rhamnoglucoside). Two mechanisms can explain these observations.

Evidence is accumulating that epithelial brush border membrane transporters play a role in the absorption of dietary (iso)flavonoids. Sodium-dependent efflux of pre-loaded radio-labelled galactose by rat everted jejunum sacs was shown to be increased when the serosal solution contained flavonoid glycosides, providing evidence that the sodium-dependent glucose transporter-1 (SGLT-1) interacts with flavonoid glycosides including quercetin-3-glucoside (Q3Glc, isoquercitrin) and quercetin-4'-glucoside (Q4'Glc, spiraeoside) [30]. Further studies have shown that uptake of methyl-α-D-glucopyranoside (a non-metabolisable glucose analogue) was inhibited competitively in the presence of Q3Glc and Q4'Glc, but not in the presence of quercetin aglycone or quercetin rutinoside [31]. Other studies using isolated rat intestines have shown that flavonoid glucosides are transported from the apical to basolateral compartment more rapidly than quercetin aglycone [32], and the transport of Q3Glc via SGLT-1 has been confirmed using rat intestine and "Ussing" chambers [33]. However, Q4'Glc was shown not to be absorbed across CaCo-2 cell monolayers, and it was demonstrated that this was due to a greater rate of basolateral to apical flux compared to that in the apical to basolateral direction [34]. Indeed, the non-absorption of Q4'Glc across CaCo-2 cell monolayers was due to efficient efflux transport, most likely due to the apical multi-drug resistance protein-2 (MRP-2) [35], and it was demonstrated that absorption of Q4'Glc into CaCo-2 cells did involve SGLT-1 [36]. Similar observations have been made in studies of the absorption of genistin (genistein-7-glucoside) across CaCo-2 cell monolayers [37].

A second route that could explain the facilitated absorption of (iso)flavonoid glucosides from the small intestine requires exogenous (luminal) deglycosylation by β-glucosidases, or co-operativity between epithelial transporters and intracellular β-glucosidases. Early work using cell-free extracts obtained from human liver and small intestine [38] or rat small intestine [39] indicated that β-glucosidase activity towards flavonoid glucoside was present in these tissues. Day and co-workers (1998) used β-glucosidase inhibitors to distinguish between the various possible enzymes, and concluded that the broad-specificity cytosolic β-glucosidase (CBG; see [40] for review) was likely to be involved. Subsequent reports have detailed the specificity (towards (iso)flavonoid glucosides) of mammalian CBGs isolated from pig liver [41] and human liver [42]. An interesting feature of CBG is that it appears incapable of hydrolysing 3-linked glucosides (e. g. Q3Glc), hence necessitating the existence of another β-glucosidase with the required specificity. Lactase-phlorizin hydrolase (LPH), specifically located to the intestinal epithelial cells, was a good candidate, based on the structural similarity between one of its known substrates, phlorizin (a dehydrochalcone glucoside), and (iso)flavonoid glucosides. Subsequently, LPH isolated from sheep small intestine was shown to efficiently hydrolyse a range of (iso)flavonoid glucosides including Q3Glc and Q4'Glc [43].

The aim of this study is to isolate and identify the human small intestinal β-glucosidases that are involved in the absorption and metabolism of dietary (iso)flavonoid glucosides. β-Glucosidases were isolated from human small intestinal mucosa using a variety of chromatographic techniques based on their ability to hydrolyse flavonoid glucosides. The enzymes were identified based on their chromatographic behaviour, cross-reaction with specific antibodies, molecular mass, and substrate specificity. The specificity of the enzymes was compared with available pharmacokinetic data to evaluate the relationship between the specificity of these β-glucosidases and the absorption kinetics for (iso)flavonoid glucosides. These data show that absorp-

tion involves deglycosylation by the luminally-exposed LPH and potentially also by the broad-specificity CBG. Only substrates for these human β-glucosidases show significant absorption from the small intestine while non-substrates are absorbed later (from the colon) and exhibit reduced bioavailability.

Materials and methods

Materials

Quercetin glycosides were obtained commercially [Extrasynthèse (ZI Lyon Nord, BP 62, 69730 Genay, France), Apin Chemicals Ltd. (Milton Park, Abingdon, Oxford, UK)], by isolation from plants [quercetin-3-xyloside (Q3Xyl), malonylated quercetin-3-glucoside (Q3GlcMal), kaempferol-3-glucuronide (K3GlA)], or biosynthetically [quercetin-3-glucuronide (Q3GlA)]. ρ-Nitrophenol-β-D-glucopyranoside was obtained from Sigma-Aldrich Ltd (Poole, Dorset, UK). Purified LPH from sheep small intestine was a kind gift from Juan C Diaz and Xavier Cañada (Instituto de Química Orgánica, CSIC, Madrid, Spain). The protease inhibitor cocktail was obtained from Sigma (product #P2714) and contained 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin, aprotinin and Na-EDTA.

Preparation of a lactase immunoaffinity column

Monoclonal antibodies specific for human lactase (mlac1, mlac6, mlac10) were prepared as described elsewhere [44]. Crude monoclonal antibodies were separated from culture media using a HiTrap Protein A column (Amersham Pharmacia Biotech; 5 ml volume) with 1.5 M glycine (pH 8.9)/3 M NaCl as the binding buffer and 0.1 M sodium citrate (pH 6.0) as the elution buffer, at a flow rate of 5 ml min^{-1}. The monoclonal antibodies were subsequently concentrated using a stirred cell with a 10 kDa cut-off membrane and used to produce an immunoaffinity column by coupling with CNBr-activated sepharose 4B (Amersham Biosciences, Little Chalfont, Bucks, UK) according to the manufacturer's instructions.

Preparation of cell-free extracts and isolation of flavonoid glucoside β-glucosidases from human small intestine

Samples of human small intestine (jejunum) were obtained from the Anatomical Gift Foundation (Laurel, Maryland, USA). The tissues were obtained fresh and snap-frozen in liquid nitrogen before use. The inter-individual variation study was performed using individual samples of ~ 2 g fresh weight intestine. The purification of enzymes hydrolysing flavonoid glucosides (Q3Glc, Q4'Glc) was performed using 50 g (fresh weight) of intestine. These procedures were performed at 1–5 °C throughout. The intestine was thawed, washed with ice-cold PBS (phosphate-buffered saline), and the mucosa removed by scraping and subsequently suspended in 3 x vol homogenisation buffer (50 mM sodium phosphate pH 7.0/1 mM EDTA/5 mM DTT/1 mM $CaCl_2$/10 mM $MgCl_2$) containing a protease inhibitor cocktail (Sigma). Homogenisation was performed using a Polytron PT 3100 homogeniser (Brinkman Instruments Inc., Westbury, NY); 15,000 rpm, 5 x 10 s bursts with 1 min cooling periods. The homogenate was centrifuged (48,000 x g, 180 min) and the supernatant (S_{48}) and pellet (P_{48}) fractions separated. For measurements of soluble and membrane-bound β-glucosidase activities, P_{48} fractions were resuspended in homogenisation buffer. For isolation of flavonoid glucoside β-glucosidases, the pellets were treated as described below.

Solubilisation of membrane-bound proteins

The P_{48} fraction was treated either with homogenisation buffer containing 4 % (w/v) Triton X-100 (pellet resuspended and incubated for 60 min with regular agitation), or with the protease papain (40 min) according to the method described in [45]. Following either treatment, the solubilised pellets were centrifuged (48,000 x g, 180 min) and the supernatants retained.

Gel filtration chromatography

Soluble proteins were applied to a column (2.6 x 100 cm) packed with sephacryl S-200 gel filtration media (Amersham Biosciences) previously equilibrated in elution buffer (10 mM sodium phosphate pH 6.0; S_{48}, $P_{48\text{-papain}}$) or elution buffer supplemented with 4 % Triton X-100 ($P_{48\text{-Triton}}$). Proteins were eluted isocratically using the same buffer at a flow rate of 13.5 cm h^{-1} (1.2 ml min^{-1}), and fractions (10 ml) collected.

Anion-exchange chromatography

Samples were applied to a column (20 x 2.6 cm) packed with DEAE-cellulose (Sigma) previously equilibrated in sodium phosphate buffer (pH 6.5). Bound proteins were eluted with a gradient of increasing sodium chloride (0–1 M) in elution buffer and fractions (5 ml) collected.

Chromatofocussing

Samples were concentrated to ~ 1 ml using a stirred cell containing a 10 kDa cut-off polyethersulfone mem-

brane, and the buffer was exchanged to 25 mM Bis-Tris/HCl (pH 6.8) using a pre-packed column containing sephadex G-25 (NAP10; Amersham Biosciences) previously equilibrated with this buffer. The sample was loaded onto a chromatofocussing column (Mono P HR 5/20; Amersham Biosciences) previously equilibrated with 25 mM Bis-Tris/HCl (pH 6.8). The column was eluted with a 5 % solution of Polybuffer 74 (Amersham Biosciences) (pH 4.0) and fractions (1 ml) collected.

Hydrophobic interaction chromatography

Ammonium sulphate was added to samples to a final concentration of 1 M. Samples were applied to a HiTrap® HR phenyl sepharose HP column (1 ml; Amersham Biosciences) and the column washed with binding buffer (50 mM sodium phosphate, pH 6.0/1.7 M ammonium sulphate/1 mM EDTA). Bound proteins were eluted using a gradient of decreasing ammonium sulphate concentration (1.7–0.0 M) in sodium phosphate buffer and fractions (1 ml) collected and assayed for β-glucosidase activity.

Affinity chromatography on octyl sepharose

Soluble proteins were loaded onto a column (1.5 × 5 cm) of octyl sepharose previously equilibrated with 20 mM sodium phosphate buffer (pH 6.5) containing 1 mM EDTA and 0.5 mM DTT, the column was washed with 20 % ethylene glycol in sodium phosphate buffer and unbound material discarded. Bound material was eluted with ethylene glycol (50 % v/v) in binding buffer at a flow rate of 0.5 ml min^{-1} over 1 h. Fractions (1 ml) were collected and assayed for β-glucosidase activity.

Immunoaffinity chromatography

Lactase was isolated specifically from samples using an immunoaffinity column (vol ~ 1 ml) containing monoclonal antibodies that recognise human lactase epitopes [44, 46]. Protein samples were diluted 10-fold with binding buffer (25 mM sodium phosphate, pH 7.5/0.15 M NaCl/4 % Triton X-100/10 mM EDTA) and applied to the column. The sample was recirculated at 0.5 ml min^{-1} for 60 min at room temperature and overnight at 4 °C. Unbound protein was removed and the column was washed with binding buffer (5 x column vol). LPH was eluted with a 25 mM sodium citrate buffer (pH 3.5) containing NaCl, Triton and EDTA as in the binding buffer. Fractions were collected and assayed for lactase activity.

Small intestinal cell culture models

TC-7 cells, a subclone of Caco-2, were kindly provided by Dr Paul Sharp (School of Biomedical and Life Sciences, University of Surrey, Guildford, UK) with the kind permission of Dr Edith Brot-Laroche (INSERM U505, Institut Biomedical des Cordeliers, Paris, France). TC-7 and CaCo-2 cells were maintained on 10 cm Petri dishes at 37 °C using Dulbecco's Modified Eagle Media (DMEM) containing 10 % foetal bovine sera.

For β-glucosidase assay experiments, CaCo-2 cells were cultured for a period of 27 days. Medium was removed by aspiration, the cells were washed twice with PBS, and 2 ml of 0.25 % trypsin/1 mM EDTA solution was added. After coating the surface of the cells, excess trypsin solution was removed. Following incubation at 37 °C for 10 min, the cells were removed by suspension in PBS using a pipette. The suspended cells were centrifuged (3,000 g x 5 min), and the supernatant discarded. Homogenisation buffer (50 mM sodium phosphate, pH 6.5/1 mM EDTA/10 mM DTT) containing a cocktail of protease inhibitors (Sigma) was added (1 ml), and the cells resuspended. Following transfer to microfuge tubes, and addition of 5 glass beads (Ø = 2 mm), cells were homogenised using a bench-top whirly-mixer type agitator (5 x 30 s bursts with 1 min cooling between bursts). Crude homogenates were assayed for flavonoid glycoside hydrolase activity (using Q3Glc and Q4'Glc as substrates), lactase activity (using lactose), and phlorizin hydrolase activity (using phlorizin).

For metabolism experiments, CaCo-2 cells were cultured until differentiation. Cells were incubated with quercetin glycosides (Q3Glc, Q4'Glc) for 4 and 24 h. The medium was removed for analysis. The cells were washed and removed from the plates as described above. The cells were homogenised and the cellular contents analysed. Both the cellular contents and the media were freeze dried and extracted using acidified methanol, and the extracts analysed for flavonoid composition using an HPLC-DAD method [47].

For transport studies, TC-7 cells were incubated on "Transwell" inserts (Corning, New York USA) to form monolayers of differentiated cells. Monolayer integrity was checked by measuring transepithelial electrical resistance (TEER) using a Millipore unit (Wathord, UK). TEER measurements were typically over 400 Ω/cm^2. Flavonoid glycosides (Q3Glc, Q4'Glc) or phlorizin (100 μM) were added to the apical compartment and the cells incubated. Apical and basolateral solutions were removed after 4 and 24 h and analysed for phenolic composition by HPLC [47].

Stable canine kidney cells expressing full-length human lactase

The procedures used to isolate the stable Madin-Darby canine kidney (MDCK) cells expressing a full-length human *LCT* cDNA (MDCK-LPH) and its properties have been described [48]. MDCK cell lines were maintained

on DMEM media containing 10 % foetal bovine serum and were sub-cultured every 2 days.

For lactase assay experiments, cells were cultured until 5-days post-confluence. Medium was removed by aspiration, the cells were washed twice with PBS, and PBST was added. After incubation for 10 min at 37 °C, the PBST was removed, PBS was added (5 ml), and the cells suspended by physical scraping. The suspended cells from 3 plates were combined, centrifuged (3,000 g x 5 min), and the supernatant discarded. Homogenisation buffer (50 mM sodium phosphate, pH 6.5/1 mM EDTA/10 mM DTT/4 % Triton X-100) containing a cocktail of protease inhibitors was added (1 ml), and the cells resuspended. Following transfer to eppendorfs, and addition of 5 glass beads (Ø = 2 mm), cells were homogenised using a bench-top whirly-mixer (5 x 30 s bursts with 1 min cooling between bursts). Crude homogenates were used for enzyme assays.

Enzyme assays

Fractions generated during isolation of proteins were routinely assayed for β-glucosidase activity using a spectrophotometric assay where the release of 4-nitrophenol (4NP) from 4-nitrophenyl-β-D-glucopyranoside (pNPGlc; 10 mM) in 50 mM sodium-phosphate buffer (pH 6.5) at 37 °C was determined at 400 nm using the molar extinction coefficient for 4NP of 18 300 M^{-1} cm^{-1}. Activities towards (iso)flavonoid glucosides and phlorizin (phloretin-2'-glucoside) were determined by measuring the amount of aglycone released from the substrate (200 µg/ml in 50 mM sodium-phosphate buffer), with particular care taken to ensure complete solubility of substrates [42]. Briefly, pure substrates were dissolved in a small volume of dimethylsulphoxide (DMSO) prior to dilution with assay buffer (50 mM sodium-phosphate buffer, pH 6.5; final concentration DMSO < 2 %, v/v), equilibrated at 37 °C, and reactions started with addition of enzyme in a final volume of 100 µl. Reactions were terminated by the addition of acetonitrile:0.1 % aq. trifluoroacetic acid (50:50, v/v; 100 µl), filtered and analysed by reversed-phase HPLC-DAD using a LUNA C-18 column (4.6 x 25 mm, 5 µm; Phenomonex, Macclesfield, UK) and an injection volume of 20 µl. Solvents A (water:tetrahydrofuran:trifluoroacetic acid, 98:2:0.1 v/v) and B (acetonitrile) were run at a flow rate of 1 ml min^{-1}. The column was re-equilibrated (5 min) in starting solvent conditions following gradient development. Standard curves were constructed using HPLC grade aglycones from which response factors were calculated and used to estimate the amount of product released in test incubations. Lactase activities were determined by measuring the amount of glucose released from α-lactose (28 mM final concentration) using an enzyme-linked assay combining glucose oxidase, peroxidase and *o*-dianisidine (GAGO-20 kit; Sigma) that gives a coloured product (λ_{max} = 540 nm). Incubations were performed in 50 mM sodium phosphate buffer (pH 6.0) at 37 °C in a final volume of 200 µl. Reactions were terminated by boiling (5 min) and allowed to cool before analysis of samples (10–100 µl) for glucose content. A glucose standard curve was treated concurrently with test samples for quantification purposes. Total protein in crude and purified samples was estimated using the Pierce Protein Assay Reagent with BSA as standard.

Analytical HPLC

A modified version of a previously published analytical HPLC method was used [47]. The analysis was carried out by reverse-phase HPLC using a Hewlett Packard 100 model. Data were collected and analysed using Chemstation software. Solvents A (water:tetrahydrofuran:trifluoroacetic acid 98:2:0.1, v:v:v) and B (acetonitrile) were run at a rate of 1 ml min^{-1}, using a gradient of 17 % B (2 min), increasing to 25 % B (5 min), 35 % B (8 min), 50 % B (5 min) and then to 100 % B (5 min). A column clean up stage was performed at 100 % B (5 min) followed by a re-equilibration at 17 % B (15 min). The column was packed with Prodigy 5 µm ODS3 reversed-phase silica, 250 mm by 4.6 mm id (Phenomenex, Macclesfield, UK). A guard column, 30 mm by 4.6 mm i. d., was also used to protect the column because the majority of samples passed through the column contained biological material. The eluent was monitored at 270 nm and 370 nm with a DAD.

Gel electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 10 % Bis-Tris pre-cast NuPAGE gels (Invitrogen, La Jolla, USA) with biotinylated marker proteins (New England Biolabs). Proteins were transferred to nitrocellulose membranes by semi-dry blotting. For immunodetection of CBG, the blots were probed with a 2000-fold dilution of polyclonal antiserum raised in rabbits against CBG from human liver (in house preparation). For immunodetection of LPH, the blots were probed with a 500-fold dilution of mouse anti-human LPH monoclonal antibody (mlac1). Immunoreactive proteins were visualised using a horseradish peroxidase anti-rabbit or anti-mouse secondary antibody (Sigma, 1:2000) together with the chemiluminescent detection reagents (ECL Plus Detection Kit; Amersham Biosciences).

Results

Isolation and identification of flavonoid glucoside β-glucosidases from human small intestine

Human small intestine was homogenised and separated into an insoluble (P_{48}) and a soluble (S_{48}) fraction. Both fractions were shown to contain significant β-glucosidase activity, but with the majority (70 % of pNPGlc activity) in the sedimented fraction. Over 90 % and 70 % of the β-glucosidase activity was associated with the sedimented fraction, for Q3Glc and Q4'Glc respectively.

Identification of flavonoid glycoside β-glucosidases in the soluble fraction

Fractionation of the S_{48} sample using gel filtration chromatography yielded four peaks of β-glucosidase activity (Fig. 2). Peaks A, B and C contained activity towards pNPGlc, Q4'Glc, Q3Glc and lactose. Peak D contained activity towards pNPGlc and Q4'Glc only. Fractionation of peak D using anion-exchange, chromatofocussing, and hydrophobic interaction chromatography always generated a single peak of β-glucosidase activity (pNPGlc or Q4'Glc as substrate; data not presented). Over 99 % of the β-glucosidase activity in peak D bound tightly to a CBG affinity column (octyl sepharose) and could be eluted with polyethylene glycol, and hydrolysed Q4'Glc but not Q3Glc, providing evidence that the β-glucosidase activity in this fraction was due to CBG (see [42]). Furthermore, the β-glucosidase eluted from the octyl sepharose column with polyethylene glycol was shown to contain a protein that cross-reacted with anti-human CBG polyclonal antibodies and had similar migration properties on SDS-PAGE to a highly purified recombinant human liver CBG (Fig. 3). Peaks A–C all eluted earlier from the gel filtration column than CBG and hence were of higher apparent molecular weight. Peaks A–C possessed similar ratios of activity towards pNPGlc and lactose, hydrolysed phlorizin and Q3Glc, and were good candidates to be full-length lactase and truncated derivatives, possibly produced through the action of luminal protease activity. A pool of peaks A–C bound to, and could be eluted from, a human lactase immunoaffinity column. The presence of lactase in a pooled sample containing peaks A–C was confirmed by immunodetection of proteins separated by SDS-PAGE (data not presented).

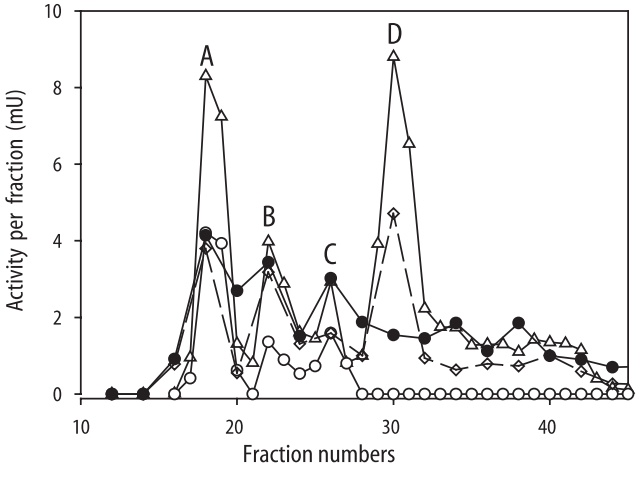

Fig. 2 Gel filtration chromatography of soluble proteins derived from a crude cell-free extract of human small intestinal mucosa. The supernatant obtained following sedimentation (48,000 x g, 3 h) of crude cell-free extracts from human small intestinal mucosa was fractionated on a gel filtration column (sephacryl S-200 HR) and fractions assayed for β-glucosidase activity using pNPGlc (◊), lactose (●), Q3Glc (○), ■ and Q4'Glc (Δ) as substrates

Identification of flavonoid glycoside β-glucosidases in the insoluble fraction

The β-glucosidase activity in the P_{48} fraction could be solubilised by treatment with either papain ($P_{48\text{-papain}}$) or Triton X-100 ($P_{48\text{-Triton}}$). Treatment of P_{48} with papain solubilised 46 % of the flavonoid glycoside hydrolase activity, whereas treatment with triton ($P_{48\text{-Triton}}$) solubilised 79 % of the activity (Q4'Glc as substrate). Separation of $P_{48\text{-papain}}$ by gel filtration chromatography generated a single peak of β-glucosidase activity. A pool of the active fractions from gel filtration chromatography demon-

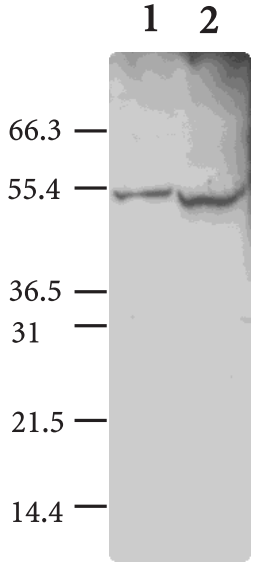

Fig. 3 Immunoblotting analysis of human small intestinal cytosolic β-glucosidase. Samples of partially purified human small intestinal CBG (following gel filtration and affinity chromatography) and highly purified recombinant CBG were separated using electrophoresis with a 10 % homogeneous polyacrylamide gel, blotted to a nitrocellulose membrane, and subsequently probed for CBG using immunodetection. Lane 1, 0.1 µg of pure recombinant human CBG; lane 2, 0.5 µg semi-purified small intestinal CBG. The positions of biotinylated molecular weight marker proteins that were run concurrently with the samples are indicated on the left

strated activity towards pNPGlc, lactose, phlorizin, Q3Glc and Q4'Glc. The active fractions obtained from gel filtration chromatography of $P_{48\text{-papain}}$ were fractionated further using several chromatographic techniques including anion-exchange, hydrophobic interaction and chromatofocussing. In each case, a single peak of activity towards flavonoid glucosides was observed (data not presented). However, due to poor yields, we were not able to isolate sufficient protein for analysis by SDS-PAGE and immunodetection. Separation of $P_{48\text{-Triton}}$ using gel filtration chromatography yielded two peaks of β-glucosidase activity (pNPGlc as substrate), but only one of these demonstrated activity towards flavonoid glucosides (data not presented). The flavonoid glucoside β-glucosidase activity was shown to bind to an immunoaffinity column comprising immobilised anti-human lactase monoclonal antibodies (mlac1). Lactase activity (lactose substrate) could be eluted from the immunoaffinity column by reducing the pH. The immunoaffinity-purified sample gave four discrete bands on SDS-PAGE, each of which was shown to cross react with monoclonal antibodies directed against anti-human lactase (Fig. 4). Solubilised human small intestine fractions have previously been shown to be highly susceptible to generating multiple bands detectable upon analysis by SDS-PAGE and immunoblotting (Dallas Swallow, University College, London, UK; personal communication). The major Coomassie-stained protein band corresponded to an apparent molecular weight of 170 kDa, similar to that for full length human LPH [46].

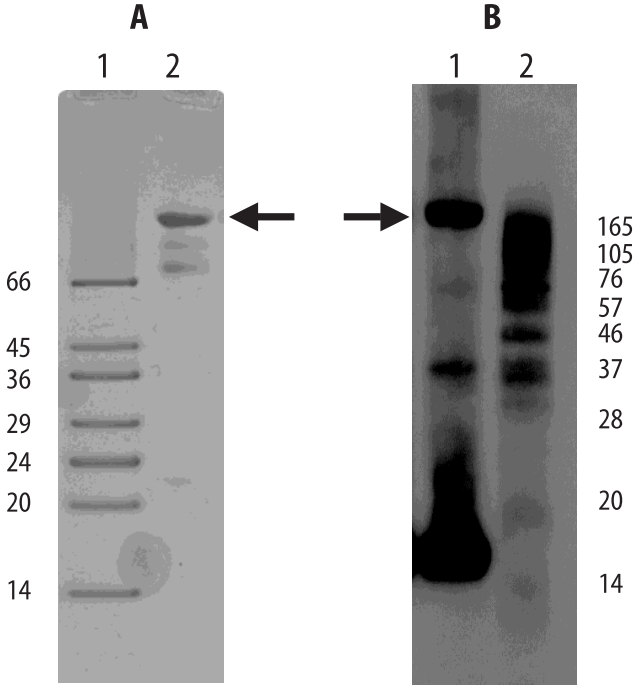

Fig. 4 SDS-PAGE and immunoblotting analysis of human small intestinal LPH. Samples of partially purified human small intestinal LPH (following gel filtration and immunoaffinity chromatography) were separated using electrophoresis with a 10 % homogeneous polyacrylamide gel and subsequently either (**A**) stained with coomassie blue or (**B**) blotted to a nitrocellulose membrane and probed for LPH using immunodetection. **A** Lane 1, molecular weight marker proteins; lane 2, purified human small intestinal LPH. **B** Lane 1, purified human small intestinal LPH; lane 2, biotinylated molecular weight markers. The position of full length LPH is indicated with arrows

Inter-individual variation in flavonoid glucoside β-glucosidase activity in human small intestine

Samples of human small intestine derived from 10 individuals were analysed for their β-glucosidase activity, and specifically their capacity to hydrolyse flavonoid glucosides. Cell-free extracts were prepared and incubated with pNPGlc, Q3Glc, Q4'Glc, and phlorizin. There was considerable variation in β-glucosidase activity between the individuals (Table 1). There was a ~ 35-fold variation in the specific activities towards pNPGlc, a measure of general β-glucosidase activity, and even greater variation with respect to the other substrates (up to 87-fold for hydrolysis of Q4'Glc). The lower variation observed for hydrolysis of pNPGlc compared to flavonoid glucosides and phlorizin may be due to the existence of β-glucosidases that exhibit activity towards pNPGlc but not the other substrates.

Deglycosylation of flavonoid glucosides by small intestinal cell models and metabolism studies

CaCo-2 cells are derived from a human colon adenocarcinoma but post-differentiation exhibit many properties typical of small intestinal epithelial cells. To assess whether differentiated CaCo-2 cells were able to metabolise flavonoid glucosides, cells were cultured until fully differentiated, and then incubated with Q4'Glc over 4 and 24 h. No loss of substrate or appearance of quercetin aglycone or metabolites (glucuronides, sulphates) was detected. Neither was metabolism observed when the cells were co-incubated with cAMP (a known

Table 1 Inter-individual variation in the β-glucosidase activities of human small intestinal mucosa. Cell-free extracts were prepared using mucosa removed from samples (~ 2 g) of human small intestine (jejunum and duodenum) derived from 10 individuals. Samples of cell-free extract were incubated with each substrate (in triplicate) in 50 mM sodium phosphate buffer (pH 6.5) at 37 °C. Data are specific activities expressed as μmol substrate hydrolysed min^{-1} (g fresh weight tissue)$^{-1}$

Substrates	Mean	Standard deviation (n = 10)	Range	Variation (fold)
pNPGlc	0.450	0.450	0.05–1.65	35
Phlorizin	0.241	0.365	0.0151–1.250	83
Q3Glc	0.096	0.134	0.0067–0.456	68
Q4'Glc	0.285	0.376	0.0151–1.320	87

up-regulator of lactase) and Q4'Glc. CaCo-2 cells were then incubated over 27 days and cell-free extracts tested for β-glucosidase activity towards Q3Glc, Q4'Glc and lactose. No activity was detected against lactose or Q3Glc at any time points. A low level of activity towards Q4'Glc was detected, equivalent to about 5 % of levels present in human small intestine. This activity is probably due to low levels of CBG in CaCo-2 cells.

TC-7 is a sub-clone of CaCo-2 that exhibits a much higher level of sucrase/isomaltase and has been reported to have a slightly higher level of lactase activity [58]. Measurements of lactase and sucrase activities made with cell-free extracts of TC-7 indicated specific activities of 0.12 and 240 μmol min^{-1} (mg protein)$^{-1}$, respectively. TC-7 cells were incubated on "Transwell" inserts and flavonoid glucosides (Q3Glc, Q4'Glc, phlorizin) added to the apical compartment. The contents of the apical and basolateral compartments were analysed for polyphenols and metabolites composition after 4 and 24 h. Q3Glc and Q4'Glc concentrations in the apical compartment were all reduced compared to the zero time point control (Fig. 5). The concentration of phlorizin was also reduced at 4 and 24 h (data not presented). The rate of loss of substrate was greatest for Q4'Glc but considerably slower for Q3Glc, with 84 % and 40 % of hydrolysis after 4 h, respectively. There was no transfer of the substrates or detectable levels of metabolites to the basolateral compartment from either Q3Glc or Q4'Glc (Fig. 5), but there was some transfer for phlorizin (data not presented). For both flavonoid glycosides, small amounts of aglycone and metabolites were present in the apical compartment. These data indicate that TC-7 cells contain measurable flavonoid glucoside β-glucosidase activity and are capable of deglycosylating flavonoid glucosides. Deglycosylation of Q3Glc and phlorizin indicates that the LPH activity present in TC-7 cells is responsible for the deglycosylation of flavonoid glycosides. However, the deglycosylation rate for Q4'Glc was considerably greater than for Q3Glc, suggesting the intracellular β-glucosidase (CBG) also plays a role in the metabolism of Q4'Glc by these cells.

Further evidence for the ability of LPH to deglycosylate extracellular flavonoid glucosides was obtained using a stable Madin-Darby canine kidney cell line that expresses full-length human LPH. These cells have been transfected with the human *LCT* cDNA and characterised [48]. The transport kinetics, post-translational processing patterns and enzymatic activities were similar to those in intestinal cells [48]. Cell-free extracts were prepared from control (non-transfected) and LPH-transfected cells and assayed for β-glucosidase activity using Q3Glc, Q4'Glc, phlorizin and lactase. No hydrolysis of lactose, phlorizin or Q3Glc was detected in control cells. Hydrolysis of Q4'Glc was observed, most likely due to the presence of CBG, which is known to be expressed in kidney tissues [40]. On the contrary, cell-free extracts prepared from transfected cells hydrolysed all four substrates (Fig. 6). These data show that membrane-bound LPH functions to deglycosylate dietary flavonoid glucosides in addition to hydrolysing lactose and phlorizin.

Specificity of mammalian LPH towards flavonoid glycosides

The ability of a highly purified LPH preparation from sheep small intestine [49] to deglycosylate flavonoid glycosides was assessed by incubating samples of the pure

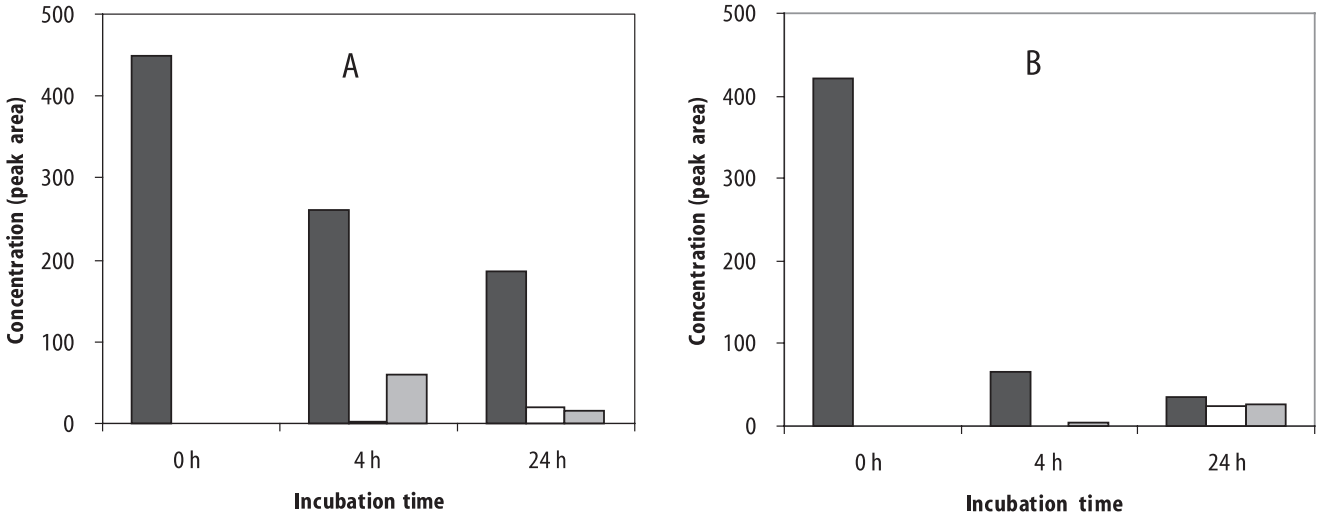

Fig. 5 HPLC analysis of TC-7 transport experiment. Cultured TC-7 cells were incubated with either **A** Q3Glc or **B** Q4'Glc for 4 and 24 h. The apical media solutions were analysed for the presence of substrates (■), aglycones (□) and metabolites (■) by HPLC-DAD. For the metabolites, the peak areas for all the glucuronides and sulfates (see [47]) were combined

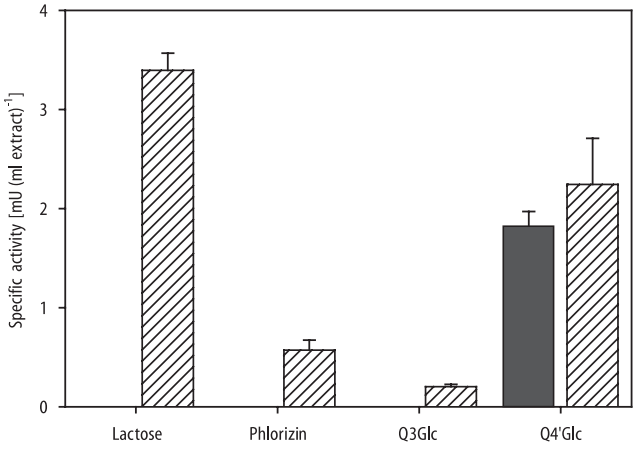

Fig. 6 Hydrolysis of flavonoid glycosides by stable canine kidney cells expressing full-length human LPH. Stable MDCK cells expressing full-length human LPH (▨) and control (non-transfected) MDCK cells (■) were used to prepare cell-free extracts and assayed for β-glucosidase activity using lactose, phlorizin, Q3Glc, and Q4'Glc. Data presented are the mean of 5 replicates, each replicate comprising the cells from 3 x 10 cm-dishes. Error bars show the standard deviation of the mean (n = 5)

Table 2 Specificity of purified sheep LPH in the hydrolysis of flavonoid glycosides

Substrate	Rate [μmol min^{-1} mg protein)$^{-1}$]
Quercetin-4′-glucoside	3.08
Quercetin-3-glucoside	3.16
Quercetin-3,4′-diglucoside (Q3Glc)	0.28
Quercetin-3,4′-diglucoside (quercetin)	0.16
Quercetin-3-galactoside	0.13
Quercetin-3-malonylglucoside	0.22
Kaempferol-3-glucoside	3.65
Kaempferol-3-glucuronic acid	0.22
Luteolin-7-glucoside	2.52
Luteolin-4′-glucoside	2.23
Luteolin-4′,7-diglucoside (release of luteolin)	0.64
Naringenin-7-glucoside	3.64
Eriodictyol-7-glucoside	3.21
Apigenin-7-glucoside	4.18
Daidzein-7-glucoside (daidzin)	0.46
Genistein-7-glucoside (genistin)	1.99
Phloretin-2′glucoside	0.22

Compounds where no hydrolysis was detected were: Quercetin rhamnoglucoside (rutin), naringin, quercetin-3-arabinoside, quercetin-3-xyloside, quercetin-3-rhamnoside, cyanidin-3-glucoside, cyanidin-3-galactoside, kaempferol-3-robinoside-7-rhamnoside, oleuropein

enzyme with putative substrates and analysing the reaction mixtures after 2 h. The enzyme was shown to hydrolyse the monoglucosides of flavonols (e. g. quercetin, kaempferol), flavones (e. g. apigenin, luteolin), flavanones (e. g. naringenin, eriodictyol), isoflavones (e. g. daidzein, genistein) in addition to the dehydrochalcone glucoside, phlorizin (Table 2). Further, LPH hydrolysed (iso)flavonoid monoglucosides that were conjugated in positions 3, 4', 5 or 7 (no substrate was available containing a 3'-glucoside moiety). LPH also hydrolysed glucuronides (e. g. kaempferol-3-glucuronide), albeit at a rate some 17-fold slower than for the corresponding glucoside (kaempferol-3-glucoside). Malonylation reduced but did not prevent hydrolysis as evidenced by a slow rate of hydrolysis of quercetin-3-malonylglucoside. Hence, LPH was capable of hydrolysing a broader range of (iso)flavonoid glucosides than CBG, mainly due to its capacity to hydrolyse 3-linked glucosides. LPH also hydrolysed Q3,4'diglucoside (a major form of quercetin in onions), producing Q3Glc and quercetin aglycone as detectable products. However, LPH was not able to hydrolyse any rutinosides such as rutin (quercetin rhamnoglucoside).

Discussion

The first step in the absorption and metabolism of dietary flavonoid glycosides involves deglycosylation by small intestinal LPH and CBG

Considerable evidence is now available supporting the hypothesis that deglycosylation is a pre-requisite to absorption of dietary (iso)flavonoid glycosides. Prior to this report however, it was not clear which enzymes were responsible, although it had been demonstrated that sheep LPH and human liver CBG were capable of hydrolysing flavonoid glycosides [42, 43]. In this report we demonstrate that only two of the β-glucosidases present in the human small intestine are capable of hydrolysing flavonoid glycosides. One of them, LPH, is a membrane bound β-glucosidase that comprises two physically similar family 1 glycosyl hydrolase domains exposed on the luminal surface of enterocytes, a trans-membrane domain, and a small, intracellular cytosolic domain [50]. The enzyme is primarily responsible for the hydrolysis of milk lactose to give galactose and glucose that are readily absorbed via sugar transporters such as SGLT-1 and members of the GLUT sugar transporter family. The second enzyme is the broad specificity cytosolic β-glucosidase (CBG), so termed due to its remarkable ability to hydrolyse a broad range of aryl glycosides including glucosides, galactosides, xylosides, arabinosides, and fucosides [40, 42]. Whereas CBG is located intracellularly, and would require active transport of hydrophilic glucosides into the cells, LPH is exposed to the lumen and its action would release aglycones into the lumen where they would be able to passively diffuse across the membrane (Fig. 7).

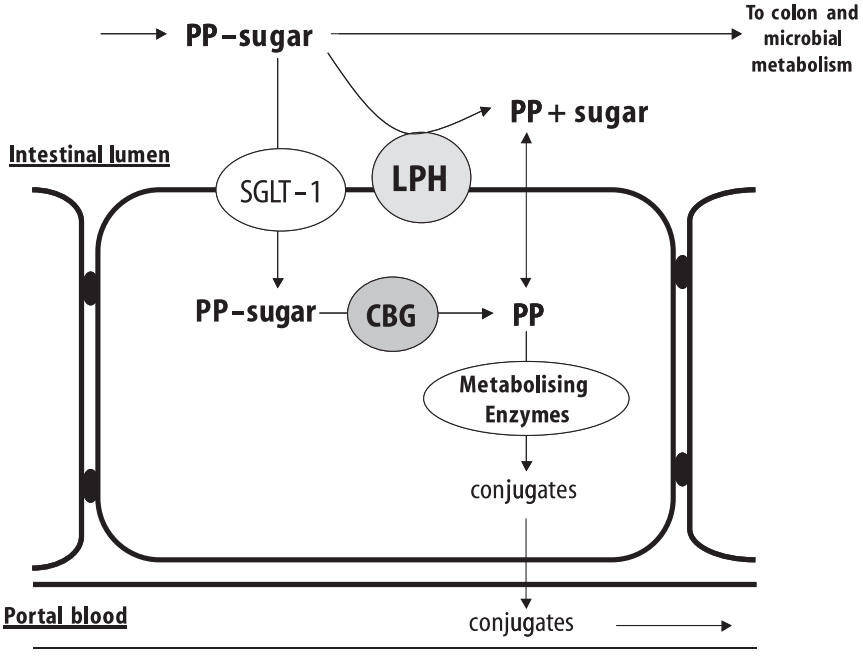

Fig. 7 A model for the absorption of dietary flavonoid glycosides highlighting the role of human small intestinal β-glucosidases. Polyphenol glycosides (PP-sugar) reach the small intestine since they are not absorbed from the stomach [25, 26]. In the small intestine they may be hydrolysed by LPH that has its catalytic sites exposed to the lumen, or transported into the enterocytes by glucose transporters such as SGLT-1 and then hydrolysed by CBG to give the aglycone (PP) which will be further metabolised into conjugates

The specificity of LPH and CBG towards (iso)flavonoid glycosides can be used to predict the bioavailability of dietary (iso)flavonoids

A detailed study of the specificity of human liver CBG towards flavonoid glycosides has been described previously [42]. Lactase isolated from sheep small intestine has also been assessed for specificity but against only a limited number of substrates [43]. In the present study, highly purified LPH isolated from sheep small intestine was further assayed using a wide range of flavonoid glycosides as putative substrates. The results demonstrated that LPH is able to hydrolyse a broad range of flavonoid glycosides including glucosides of flavonols, flavones, flavanones, and isoflavones, but was not able to hydrolyse rutinosides such as rutin (quercetin rutinoside).

Based on the specificity of the CBG and LPH β-glucosidases towards flavonoid glucosides ([42, 43]; Table 2, this report), it is possible to predict the absorption kinetics for (iso)flavonoids from a variety of foods (Table 3). Polyphenol glycosides that are substrates for LPH and/or CBG exhibit enhanced absorption kinetics and increased bioavailability. For example, onions contain high concentrations of quercetin, usually as a mixture of Q4'Glc and Q3,4'diGlc [16]. These compounds are substrates for the two human β-glucosidases that we have shown are involved in the first step in metabolism. In this example, LPH would be required to hydrolyse Q3Glc (from Q3,4'Glc). Polyphenol glycosides that are not substrates for LPH or CBG are generally not absorbed in the small intestine and will travel further down the gastrointestinal tract and reach the colon where they will be exposed to the colonic microflora and be rapidly degraded. A major flavonoid glycoside in tea is rutin (quercetin rutinoside) that is not a substrate for either LPH or CBG. We would thus predict that quercetin from

Table 3 Predicting the bioavailability of dietary flavonoids based on the specificity of human small intestinal β-glucosidases

Food source	Major flavonoid glycosides	Substrates for human β-glucosidases?	Absorption, bioavailability
Onions	Quercetin glucosides (Q3,4'diGlc, Q4'Glc)	Yes Rapidly hydrolysed	Early (small intestine), relatively high [21, 27, 29]
Tea	Quercetin rutinoside (rutin)	No	Late (colon), relatively poor bioavailability [21, 23, 29]
Apples	Quercetin monoglycosides (Q3Rha, Q3Xyl, Q3Glc, Q3Gal, Q3Ara) Quercetin rutinoside (rutin)	Only a small proportion are substrates	Intermediate [21]
Red berries (elderberries, blackcurrant)	Anthocyanins (e. g. cyanidin/delphinidin glucosides and rutinosides)	No	Very poor absorption, low levels of glucosides detected in urine [51, 52]

tea would be absorbed later than quercetin from onions, and this is supported by the pharmacokinetic data for absorption of quercetin from these dietary sources [21, 27, 29]. The exception appears to be anthocyanins since these have been detected in human urine following ingestion of blackcurrant or elderberry juice [51, 52], but only at very low levels (< 0.02 % excreted).

Inter-individual variation and polymorphisms

Previous reports of β-glucosidase activity have demonstrated the existence of considerable variation. For example, the variation in CBG activity between liver samples from different individuals was considerable, suggesting the existence of discrete CBG populations with discrete genetic identities [53]. LPH is also polymorphic, and is one of the best-known genetic polymorphisms. A deficiency of LPH is the cause of lactose intolerance that is common except in Northern European adults and a few small predominantly pastoral populations [54, 55]. Recent evidence has arisen to show that in humans, LPH is also responsible for the hydrolysis of pyridoxine-5'-β-D-glucopyranoside, a common dietary form of vitamin B-6, and this multifunctional enzyme may also play a role in vitamin B-6 bioavailability [56]. Clearly, the large variations observed in β-glucosidase activity towards (iso)flavonoid glycosides are likely to impact substantially on the ability of individuals to absorb and metabolise these dietary components.

Cultured cell models

In the present study two small intestinal cellular models were evaluated for further studies into deglycosylation as the first step of metabolism. CaCo-2 cell monolayers mimic intestinal absorptive epithelium and have been extensively used for studies into transepithelial transport and metabolism. However, they do have limitations, probably due to their origin as cancerous cells from the human colon [57]. For example, the expression levels of some proteins are markedly different from that found in healthy human tissue and animal models. In the present study the lack of detectable hydrolysis of flavonoid glycosides is probably due to the low-level of β-glucosidase activity in this particular cell type and clone. The TC-7 cell line is a sub-clone of CaCo-2 that is reported to exhibit a much higher level of sucrase activity and a slightly higher level of lactase activity than other CaCo-2 sub-clones [58]. Unlike the batch of uncloned CaCo-2 cells we were testing, TC-7 contain sufficient LPH to be suitable for metabolism studies. Neither of these cell lines are suitable for studies into the role of CBG in flavonoid glycoside absorption and metabolism since they both contain LPH and, as yet, it is not possible to distinguish between CBG and LPH since there are no known flavonoid glycosides that are substrates for CBG but not for LPH.

In conclusion, we have demonstrated that two human β-glucosidases present in the epithelial cells of the small intestine, function to deglycosylate flavonoid glycosides during passage across the gut wall. Cells that lack β-glucosidase activity do not facilitate absorption of flavonoid glycosides, and compounds that are not substrates for these β-glucosidases are not absorbed. We present some evidence that deglycosylation is a rate-limiting step for small-intestinal absorption, and hence mediates bioavailability for a large number of dietary flavonoids.

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