Hydrolysis of dietary flavonoid glycosides by strains of intestinal *Bacteroides* from humans

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Rutin and quercitrin are hydrolysed to quercetin, and robinin is hydrolysed to kaempferol, by faecal flora from healthy subjects. The enzymes required for these hydrolyses, namely α-rhamnosidase and β-galactosidase, were produced by some strains of *Bacteroides distasonis*; other strains, however, synthesized β-glucosidase. The last-named enzyme was also elaborated by *Bacteroides uniformis* and *Bacteroides ovatus*. All the enzymes were produced constitutively. A cell-free extract of *B. distasonis* containing β-glucosidase displayed an enzymic activity of 1 μmol/10 min per 10 mg of protein.

INTRODUCTION

Flavonoid glycosides are polyphenolic compounds produced by many plants. They occur in small, but significant, amounts in most fruits and vegetables. They are resistant to boiling and fermentation and are ingested in quantities of 1–2 g daily by subjects on a Western diet [1]. Flavonoid glycosides were largely ignored by nutritionists and pathologists until Brown *et al.* in 1977 [2] noted that free flavonoids or aglycones possessed potent mutagenic properties towards bacteria used in the Ames test. It is not clear whether or not the aglycones also are carcinogens or co-carcinogens. Aglycones are formed continuously in the mammalian gut by bacterial hydrolysis of ingested flavonoid glycosides [3,4]; the ensuing flavone rings are further bacterially metabolized to non-mutagenic compounds.

Of the approx. 2000 known flavonoid glycosides, quercitrin, rutin and robinin are the most common dietary compounds [5]. Quercitrin and rutin are bac-terially hydrolysed in the gut to a single product, quercetin, whereas robinin is converted into kaempferol [4].

In 1983 Macdonald *et al.* [4] reported the hydrolysis of rutin by a cell-free extract of human faecal flora ('fecalase') and of saliva ('salivase'). Furthermore, they noted β-glucosidase activity in a stock strain of the common intestinal micro-organism *Bacteroides fragilis*.

In the present paper we report on the isolation and identification of human faecal bacteria capable of hydrolysing rutin (Scheme 1) and robinin (Scheme 2) and on the ability of faecal flora to hydrolyse quercitrin.
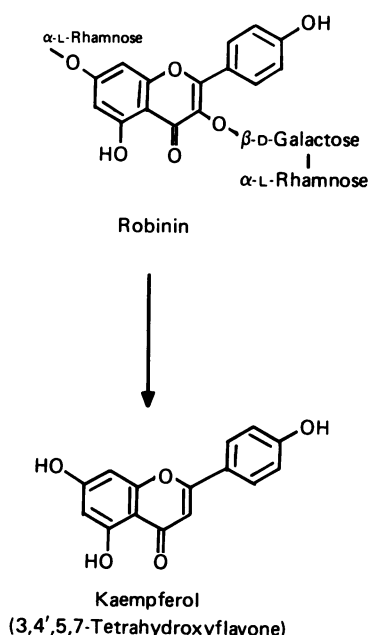
MATERIALS AND METHODS

Media

BHIC (Baltimore Biological Laboratories, Cockeys-ville, MD, U.S.A.), made up according to the manu-facturer's instructions, was supplemented with 0.5 g of
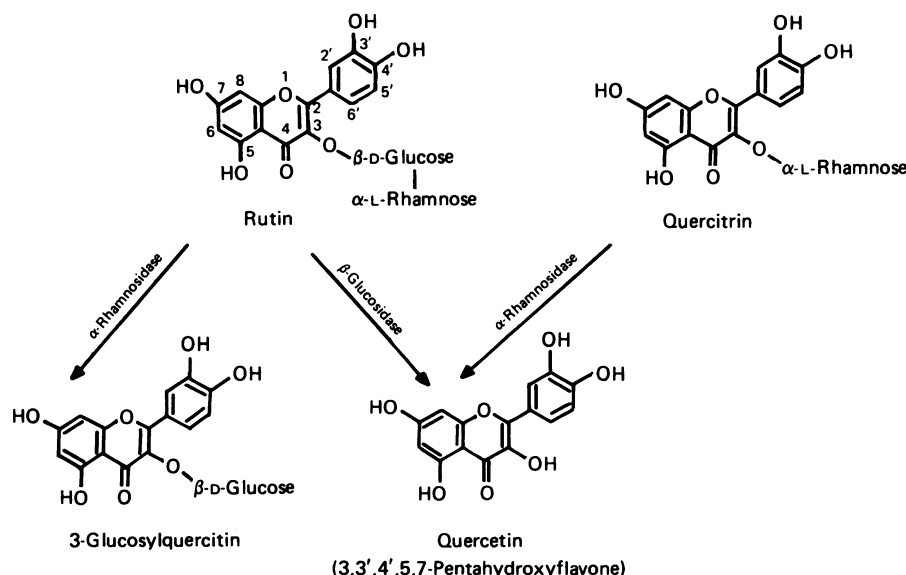
Scheme 1. Hydrolysis of rutin and quercitrin by bacterial enzymes

Robinin

Kaempferol
(3,4',5,7-Tetrahydroxyflavone)

Scheme 2. Hydrolysis of robinin to kaempferol by bacterial enzymes

cysteine hydrochloride and 1 g of $NaHCO_3$/litre. The medium was distributed in 50 ml amounts in 125 ml screw-cap vials and sterilized at 121 °C for 20 min. PR was purchased from Scott Laboratories, Fiskeville, RI, U.S.A. SPB was obtained from Becton, Dickinson and Co., Rutherford, NJ, U.S.A.; blood agar, chocolate agar and MacConkey agar plates were purchased from BBL.

Source and isolation of micro-organisms

Faecal samples from healthy subjects on a Western diet were collected in stool cups and, within 30 min, serially 10-fold diluted in SPB. Aliquots from all dilutions were tested for flavonoid-glycoside-hydrolysing bacteria (see below), and 0.1 ml samples from the three highest dilutions yielding positive results were seeded on four sets of blood-agar plates and incubated at 37 °C for 48 h, two sets aerobically and two sets anaerobically (GasPak; BBL). The colonies were counted, and growth from individual plates of one aerobic and one anaerobic set was harvested in 2.5 ml of saline (0.9% NaCl) and tested for flavonoid-glycoside-hydrolysing bacteria. The information enabled us to pin-point the sister plate, aerobic or anaerobic, with the fewest colonies that included at least one colony of flavonoid-glycoside-hydrolysing bacteria. All colonies were isolated from this plate and tested for hydrolytic activity. Pure strains of converting bacteria were identified by Dr. L. V. H. Moore and Dr. E. P. Cato, Anaerobic Laboratories, Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.

Enzymic cell-free preparations

Extracts were obtained by a modification of the method described by Macdonald et al. [6]. Instead of breaking up the organisms in a French press, we used ultrasonication (sonicator model W 370; Ultrasonic, Plainview, NY, U.S.A.; 100 W; 4×15 s at 0 °C). The protein content of the sonicated material was determined on a Gilford (Oberlin, OH, U.S.A.) 250 photocolorimeter

as described by Lowry et al. [7]. The β-glucosidase activity was determined by using the artificial substrate p-nitrophenol glycoside as described by Goldin & Gorbach [8].

Substrate and reference compounds

Rutin and quercetin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; quercitrin, robinin and kaempferol were obtained from Pfaltz and Baur, Stamford, CT, U.S.A.

Reagents and solvents

Solvents for extraction of · flavonoid glycosides/ aglycones from cultures and reference compounds for identification of products by t.l.c., g.l.c. and h.p.l.c. purchased from Sigma. Solvents were of reagent grade, except those used for h.p.l.c., which were of h.p.l.c. grade.

Conversion cultures

Before incubation, PR or BHIC (50 ml) was supplemented with 1 mg of flavonoid glycoside dissolved in 0.25 ml of methanol, giving a final concentration of 20 μg/ml. To enhance the anaerobic conditions in selected experiments, BHIC was seeded with 0.1 ml of a 24 h culture of a non-converting strain of Escherichia coli (BHIC-Ec [9]). The culture-ready medium was then seeded with 0.25 ml of progressively diluted faecal suspension (10^2–10^{11}) or 0.25 ml of a 24 h-old pure culture in SPB. After incubation of the conversion culture at 37 °C for 1–7 days, substrates and/or metabolites were extracted.

Extraction of flavonoid glycosides/aglycones

Portions of conversion cultures, 5 ml for t.l.c. or 45 ml for h.p.l.c., were extracted with ethyl acetate for 30 min. The organic phase was separated, dried over anhydrous Na_2SO_4 and evaporated under N_2 at 40–45 °C.

Identification of metabolites

(1) **T.l.c.** The extracted residue was redissolved in 50 μl of methanol and spotted on IB2F Baker Flex silica-gel plates (J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.). Good separation was obtained with benzene/ acetic acid/water (83:48:2, by vol.). The flavonoid glycoside/aglycone spots were located by exposure to u.v. light and I_2 vapour.

(2) **H.p.l.c.** This was performed on an h.p.l.c. 5000 instrument (Varian Instrument Group, Walnut Creek, CA, U.S.A.) with a Chromapack spher C_8 column (Chromapack, Bridgewater, NJ, U.S.A.) and u.v. detector at 256 nm under the following conditions: solvent system, water/acetic acid/methanol, 80:6:65 (by vol.); flow rate, 1 ml/min; column temperature, 22 °C. The products that were submitted to h.p.l.c. analysis were extracted from the cultures as described above, purified by t.l.c. and extracted from the plates with 5 ml of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 0.5 ml of the h.p.l.c. solvent system and 10 μl was injected into the column. Authentic standards were incorporated in all runs. Alternatively, the metabolic products were purified on a Sep-Pak C_{18} cartridge (Water Associates, Milford, MA, U.S.A.), followed by elution with ethyl acetate.

(3) M.s. To validate the results of t.l.c. and h.p.l.c examination, derivatized aglycone metabolites were periodically tested on a Hewlett–Packard 5970 mass-selective detector. The instrument was equipped with a 15 m OV-1-type fused-silica column. Sample introduction was by splitless injection, and the temperature was programmed from 210 to 300 °C at 3 °C/min. Data were acquired by repetitive scanning over the 100–800 mass range.

Quercetin was derivatized as follows. A 0.1 ml portion of 2% (w/v) methoxyamine hydrochloride in pyridine was added to the sample, and the mixture was heated for 1 h at 60 °C. It was then dried under N_2 and 100 μl of trimethylsilylimidazole was added. After 3 h incubation at 100 °C, the reagent was removed on a Lipidex 5000 chromatography column (Packard Instruments Co., Downers Grove, IL, U.S.A.), and the compound was eluted with cyclohexane [10]. The sample was stored in cyclohexane before analysis.

RESULTS

Enumeration and isolation of faecal flavonoid-glycoside-hydrolysing bacteria

Faecal flora from four healthy volunteers, diluted 10^8–10^9 and incubated 24 h with substrate in BHIC-Ec or PR, hydrolysed rutin to quercetin (Scheme 1) and robinin to kaempferol (Scheme 2). Quercitrin was hydrolysed by a faecal culture diluted 10^2–10^5. The chromatographic data for the flavonoid-glycoside substrates and their aglycone metabolites are shown in Table 1.

To isolate converting organisms, 0.1 ml aliquots of the most diluted suspensions containing detectable hydrolysing bacteria (10^7–10^9) were seeded on four sets of agar plates and incubated, harvested and tested for flavonoid-glycoside-hydrolysing enzymes as described in the Materials and methods section. The enzymes were found exclusively in cultures grown under anaerobic conditions. As conversion required the presence of approx. 60 colonies on blood agar plates and chocolate agar, all colonies from the corresponding sister plates were isolated, batch-tested for hydrolysing enzymes and then tested individually. We recovered three enzyme-producing strains, all of which were obligate anaerobes. They were identified as *Bacteroides distasonis*, *B. uniformis* and *B. ovatus*.

Some strains of *B. distasonis* synthesized a β-glucosidase, whereas others produced a mixture of α-rhamnosidase and β-galactosidase. The latter organism converted robinin into kaempferol and converted rutin into a monosaccharide 3-glucosylquercetin, which could be further hydrolysed to the aglycone, quercetin, by addition to the conversion culture of the β-glucosidase-producing strain.

B. uniformis and *B. ovatus* both synthesized β-glucosidase, which hydrolysed rutin to quercetin, but failed to metabolize robinin. Type strains of the two latter organisms also synthesized β-glucosidase.

Quercitrin was not hydrolysed by bacterial isolates recovered in these experiments, including those synthesizing α-rhamnosidase.

Cell-free preparations

Extracts of *B. distasonis* synthesizing β-glucosidase only were prepared as described in the Materials and methods section. The preparations contained 1 mg of

Table 1. Chromatographic data for flavonoid glycosides and their metabolites

R_F is the relative (to solvent front) mobility on t.l.c. RT is the retention time on h.p.l.c. Chromatographic details are given in the Materials and methods section. Abbreviation: ND, not done.

Flavonoid (flavonoid glycoside/aglycone)	R_F	RT (min)
Rutin	0.06	5.6
Quercitrin	0.10	7.9
Quercetin	0.44	14.0
3-Glycosylquercetin	0.10	ND
Robinin	0.03	4.5
Kaempferol	0.57	25.6

protein/180 μl and linearly hydrolysed p-nitrophenol glycoside in a volume of 50–250 μl. The specific activity was 1 μmol/10 min per 10 mg of protein. Enzyme synthesis by *B. distasonis* was not enhanced in media supplemented with 20 or 60 μg of rutin/ml.

DISCUSSION

Origin of intestinal flavonoid glucosidases

Griffiths & Barrow [3] showed in 1972 that germ-free rats ingesting flavonoid glycosides excreted the compounds in faeces in an unhydrolysed form. More recently investigators found mammalian tissues incapable of synthesizing the appropriate hydrolases [11,12]. Macdonald et al. [13], working with anaerobic stock strains of *B. fragilis*, found that one of these produced a non-inducible β-glucosidase that converted rutin into quercetin. Although *B. fragilis* is one of the most common faecal organisms in humans, it was not among the flavonoid-glycoside-hydrolysing isolates in our experiments. Nor did we succeed in demonstrating β-glucosidase activity in five recent isolates of *B. fragilis* from surgical specimens. Instead we found β-glucosidase synthesis by *B. ovatus*, *B. uniformis* and one strain of *B. distasonis*. Moreover, α-rhamnosidase and β-galactosidase were synthesized by other strains of *B. distasonis*.

It is entirely possible that, in humans, flavonoid glycosides may be hydrolysed by other, less prevalent, intestinal species, since our isolation technique favours detection of the most common organisms. Furthermore, in other mammals other bacterial species may be responsible for the hydrolysis.

Our limited experiments suggest that synthesis of flavonoid-glycoside-hydrolysing enzymes by our isolates is independent of the presence of substrate; that is, they appear to be produced constitutively.

Specificity of the enzymes

The experiments have provided evidence that at least some of the bacterial glycosidases are capable of cleaving both glycosidic bonds and flavonoid–saccharide bonds. It appears, however, that a specific enzyme is required not only for each type of conjugated carbohydrate, but, at least in certain cases, also for its position of the flavone molecule. For example, α-rhamnosidase of *B. distasonis* hydrolysed robinin, which has α-rhamnose at C-7, but not quercitrin, which has α-rhamnose at C-3. This might

well indicate that the intestinal bacteria are equipped with a vast array of enzymes for hydrolysis of the numerous dietary flavonoid-glycoside compounds.

Metabolism of flavonoid glycosides in the mammalian organism

As it has been shown that ingested flavonoid glycoside is excreted in the faeces by germ-free animals, the first step of the flavonoid-glycoside metabolism in the normal animal appears to be bacterial hydrolysis to the mutagenic aglycone. The conversion probably takes place in the lower part of the ileum and the caecum. The aglycone molecules are then either further bacterially metabolized to unknown, probably non-mutagenic, structures, or absorbed into the enterohepatic system [12]. In the liver they are conjugated as glucuronides and sulphates and delivered to the sinusoids for renal excretion [12]. The possibility exists, however, that they, like conjugated ring-A-reduced steroid hormones [3], may also undergo enterohepatic circulation, including bacterial deconjugation in the gut. The final fate of the aglycone seems to be that they are either bacterially degraded or conjugated in the liver and eliminated by renal excretion [12].

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