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SHORT COMMUNICATION

Small intestinal hydrolysis of plant glucosides: higher glucohydrolase activities in rodents than passerine birds

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ABSTRACT

Glycosides are a major group of plant secondary compounds characterized by one or more sugars conjugated to a lipophilic, possibly toxic aglycone, which is released upon hydrolysis. We compared small intestinal homogenate hydrolysis activity of three rodent and two avian species against four substrates: amygdalin and sinigrin, two plant-derived glucosides, the sugar lactose, whose hydrolysis models some activity against flavonoid and isoflavonoid glucosides, and the disaccharide sugar maltose (from starch), used as a comparator. Three new findings extend our understanding of physiological processing of plant glucosides: (1) the capacity of passerine birds to hydrolyze plant glucosides seems relatively low, compared with rodents; (2) in this first test of vertebrates' enzymic capacity to hydrolyze glucosinolates, sinigrin hydrolytic capacity seems low; (3) in laboratory mice, hydrolytic activity against lactose resides on the enterocytes' apical membrane facing the intestinal lumen, but activity against amygdalin seems to reside inside enterocytes.

KEY WORDS: Cytosolic β-glucosidase, Digestion, Enzyme, Lactase phlorizin hydrolase, Plant secondary compounds

INTRODUCTION

Plants produce a variety of glycosides, which are characterized by a lipophilic, possibly toxic aglycone conjugated to one or more sugars (e.g. glucose, in which case it is a glucoside) (Harborne, 1993). Hydrolysis of the glycosidic bond of toxic plant glycosides by a glycohydrolase enzyme is important for the bioactivation of the aglycone, its absorption during digestion and its effect(s) post-absorption (Walle et al., 2000). Small intestinal enzymes hydrolyze plant glucosides in many mammals, including humans, rats and guinea pigs (Day et al., 2000; Karasov and Douglas, 2013). Although birds may have a homolog of the lactase gene, it is uncertain whether birds are capable of hydrolyzing plant glycosides (Karasov and Douglas, 2013; Struempf et al., 1999). The cyanogenic glucoside amygdalin is toxic to rats (*Rattus norvegicus*) but not to cedar waxwings (*Bombycilla cedrorum*), apparently because it is hydrolyzed in the intestine of the former but not the latter (Struempf et al., 1999). We studied intestinal glycosidase activity against three substrates by three rodent and two avian species to test the general hypothesis that rodents would have higher activity than birds.

We compared small intestinal homogenate hydrolysis activity against the following three substrates: amygdalin and sinigrin (a β-glucoside glucosinolate), two plant-derived glucosides, and the sugar lactose, whose hydrolysis models some activity against plant glucosides. The ability of enzymes in a vertebrate consumer's gut to hydrolyze glucosinolates has not been studied previously. The three substrates are likely hydrolyzed by at least two glycosidases: the cytosolic β-glucosidase (CBG), an intracellular enzyme with broad specificity present in both the mammalian liver and small intestine (Day et al., 2000), and lactase phlorizin hydrolase (LPH), a β-glucosidase found on the apical membrane of enterocytes in the mammalian small intestine. LPH is known for its role in the hydrolysis of lactose in milk (Karasov and Douglas, 2013), but several flavonoid and isoflavonoid glucosides are also hydrolyzed by LPH (Day et al., 2000).

We studied common laboratory models – laboratory house mice (*Mus musculus* Linnaeus 1758) and house sparrows [*Passer domesticus* (Linnaeus 1758)] – and also rodent and avian members of a guild of consumers of glucosinolate-containing fruit from the desert plant *Ochradenus baccatus* in Israel. *Ochradenus baccatus* contains at least six glucosinolates including sinigrin in the fruit pulp, and in the seeds it contains myrosinase, a plant enzyme that hydrolyzes glucoinolates (Samuni-Blank et al., 2013). The common spiny mouse [*Acomys cahirinus* (Geoffrey 1803)] consumes mainly the fruit pulp, ingesting the inactive form of glucosinolates, whereas its congener the golden spiny mouse [*Acomys russatus* (Wagner 1840)] more often consumes both the pulp and seed, which it macerates, thus ingesting the activated toxic form of glucosinolates (Samuni-Blank et al., 2013). White-spectacled bulbuls (*Pycnonotus xanthopygos* (Hemprich and Ehrenberg 1833)], birds commonly found in Israel, also consume the fruit and do not macerate the seeds but either egest or excrete them more-or-less intact.

We predicted that (i) rodents would have higher constitutive levels of hydrolytic activity against the glucosides amygdalin and sinigrin as well as lactose compared with birds because of the expected relatively higher levels of LPH in mammals and based on other empirical evidence in the literature (above). As a comparator for these activities against β-glucosides, we also measured hydrolytic activity against the nutrient maltose, and predicted that (ii) rodents and birds would have a similar maltase activity that is relatively higher than activity against non-nutritive plant glycosides. We mainly used intestinal tissue homogenates to measure activity. However, to distinguish between activity localized at the apical membrane of enterocytes from that in the cytosol, we also measured activity against some of the substrates using everted sleeves of intestine that measure only the apical component (O'Connor and Diamond, 1999).

RESULTS AND DISCUSSION

Homogenate enzyme assay

Hydrolysis of maltose, which we used as a general comparator and as an index of tissue viability, was at least an order of magnitude

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higher than hydrolysis of the other substrates and in most cases not significantly different between avian and rodent species (Fig. 1A). In marked contrast, however, hydrolysis of glucosides was undetectable in the birds and was significantly lower in avian than mammalian species in many cases (Fig. 1B–D). Amygdalin and lactose hydrolysis were also undetectable in house sparrows we captured and measured in Madison (WI, USA) (supplementary material Fig. S1). Among the *Acomys* spp., hydrolysis of both lactose and amygdalin was significantly higher in the golden than in the common spiny mouse, and in the latter species, hydrolysis of amygdalin was undetectable (Fig. 1B,C). The laboratory mouse was the only species tested for which hydrolysis of sinigrin was detectable (significantly higher than zero; t_9=2.37, P<0.05). The generally higher glucosidase activity in rodents compared with birds (Fig. 1B–D) occurred despite a 3°C higher assay temperature in birds (corresponding to their higher body temperature) and did not depend on the particular standard conditions of substrate concentration and pH at which assays were run (supplementary material Fig. S1).

Everted sleeve enzyme assay in house mice

When comparing the hydrolysis of lactose by the everted sleeves with hydrolysis by the homogenates, the rates of lactose hydrolysis from the two preparations were similar (Table 1), consistent with the idea that most activity was situated at the apical membrane of enterocytes. In contrast, amygdalin hydrolysis was undetectable in everted sleeves whereas it was detected when using homogenates (Table 1). This is consistent with the idea that most amygdalin hydrolysis activity is situated in the cytosol and not the apical membrane, although the difference between tissue preparations did not achieve statistical significance.

Three new findings extend our understanding of physiological processing of plant glucosides: (1) the capacity of passerine birds to hydrolyze plant glucosides seems relatively low in most comparisons with rodents; (2) in this first test of vertebrate enzymic capacity to hydrolyze glucosinolates, sinigrin hydrolytic capacity seems low; and (3) the intestinal site of amygdalin hydrolytic capacity seems to be the cytosol.

Hydrolysis of glucosides was undetectable in birds, but this was not an artifact of our methods. The observed high rates of hydrolysis for maltose in our birds, which are comparable to maltase activity found in other studies, validate that enzymes in the small intestine were indeed viable and active. Our findings are consistent with the finding that another bird, the cedar waxwing, was not affected by the toxic glucoside amygdalin when administered orally, excreting it intact (Struempf et al., 1999). In contrast to the birds, in the rodents, glucosides were hydrolyzed by intestinal homogenates, though we were clearly at the limits of detection of sinigrin hydrolysis. A confounding feature of our study was that we did not use diets with and without glucosides, and the dissimilar diets of the rodents and birds may have included different amounts of glucosides. ß-Glucosidase activity is reduced in some insects that have been habituated to diets with higher levels of glycosides (Karasov and Douglas, 2013). These kinds of studies have not been done in mammals and birds, but future studies should take account of possible effects of diet.

Several flavonoid and isoflavonoid glucosides are hydrolyzed at the apical membrane of enterocytes by LPH (Day et al., 2000). Amygdalin is a cyanogenic glucoside, and our experiments with amygdalin hydrolysis by house mice (Table 1) showed that it was not significantly hydrolyzed by the apical membrane of intestinal sleeves, where LPH resides, even though amygdalin could be

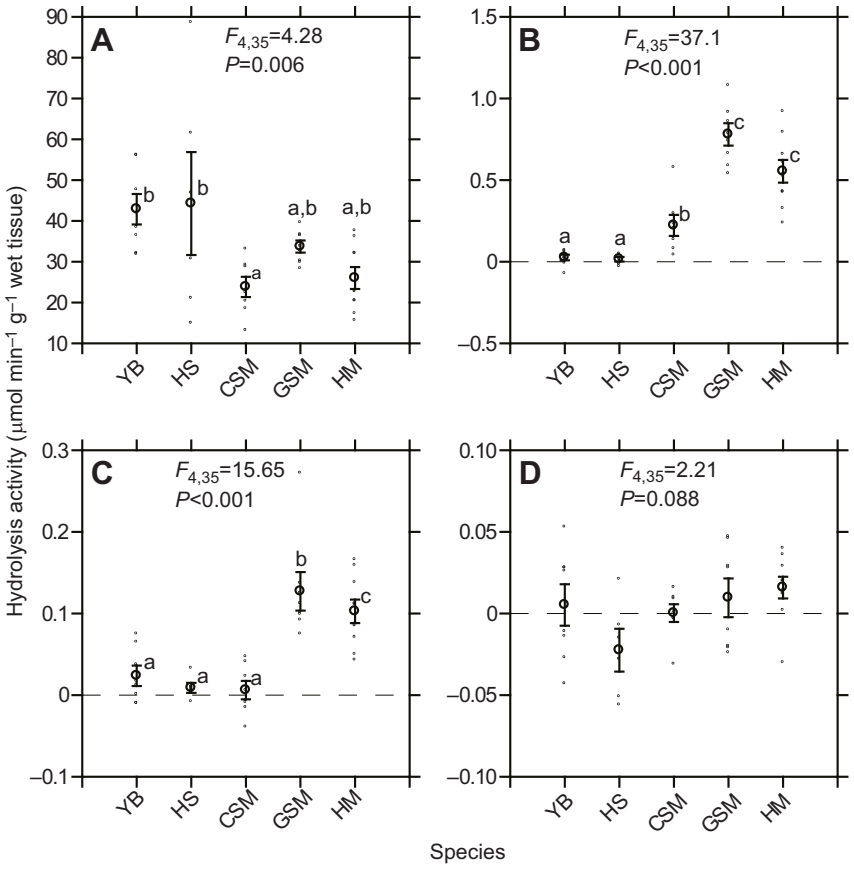

Fig. 1. Hydrolysis activity of four substrates by intestinal homogenates from five species. (A) Maltose, (B) lactose, (C) amygdalin and (D) sinigrin. The species include yellow-vented bulbuls (YB), house sparrows (HS) common spiny mice (CSM), golden spiny mice (GSM) and laboratory (house) mice (HM). Within each plot, the open circles and bars represent the mean±s.e.m. for the particular species, and the dots are the actual values (N=8–10 individuals/species). Some values are negative for reasons described in Materials and methods. For each substrate, the one-way ANOVA result is provided for comparison among species, and bars that do not share the same lowercase letter are significantly (P<0.05) different from each other in a Bonferroni pair-wise comparison.

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Table 1. Comparison of hydrolysis of β-glucosides by two tissue preparations from house mouse intestine

	Hydrolysis activity (μmol min^{-1} g^{-1} wet mass)	
Preparation	Lactose	Amygdalin
Tissue homogenates	0.548±0.069 (10)*	0.1022±0.0143 (10)*
Everted sleeves	0.504±0.079 (10)*	0.0282±0.0387 (10)
$F_{1,18}$-value	0.18	3.85
P-value	0.68	0.075

Data using homogenates reflect intracellular and apical membrane-derived activity, whereas those using everted sleeves reflect activity at the apical membrane (means±s.e.m.). Number of samples is given in parentheses.
F- and *P*-values are for comparison between preparations.
*Mean value significantly greater than zero.

hydrolyzed by intestinal homogenates. Thus, amygdalin hydrolysis may be via activity of CBG. CBG activity might also occur in the lumen, which we did not assay, either from intestinal cells that have been sloughed off from the villi and are breaking down, or from luminal microbes (Hanske et al., 2011). These possibilities remain to be explored, but the data so far suggest why rodents would likely be more affected by amygdalin than passerine birds. Also, rodents that are hindgut fermenters are expected to have a higher capacity for microbial biotransformation of glucosides than passerine birds, which mostly lack an enlarged hindgut fermenting chamber (Karasov and Douglas, 2013).

The low or nil capacity of passerine birds to hydrolyze plant glucosides might make them relatively immune to these plant toxins compared with other animals, although we studied only two species and only a few types of substrate. There are other examples of physiological differences between animals in processing plant secondary compounds, and they likely have ecological significance (Karasov and Douglas, 2013). The sensation(s) induced by irritants such as capsaicin deters mammals but not birds, perhaps because the avian homolog of the vanilloid receptor does not interact with capsaicin (Jordt and Julius, 2002). This kind of differential response between birds and mammals, like that described above for the response to amygdalin (Struempf et al., 1999), might be considered consistent with the 'directed toxicity hypothesis', which posits that secondary compounds in ripe fruit are toxic (or deterrents) for vertebrate fruit consumers that do not disperse viable seeds, but have no or little toxic effect on seed-dispersing frugivores (Jordt and Julius, 2002). Because plant toxins mediate so many interactions between mammals and birds and their plant resources (e.g. leaf, fruit and seed diet selection, seed and pollen dispersal), physiological differences among them in their responses to toxins should have many ecological ramifications, but the details of most remain to be explored.

MATERIALS AND METHODS

Animals and their maintenance

Common/Egyptian spiny mouse (*A. cahirinus*), golden spiny mouse (*A. russatus*), yellow-vented bulbul (*P. xanthopygos*) and house sparrows (*P. domesticus*) were trapped at various locations in Israel under permit (Israel Nature and National Parks Authority). Rodents were housed in pairs in plastic cages (21×31×13 cm) under constant environmental conditions (25±2°C, relative humidity of 35±3%) and a lighting schedule of 12 h:12 h light:dark, and had access to *ad libitum* rodent chow (Koffolk serial no. 19510) and fresh carrots as a source of free water. Birds were housed in a large open outdoor cage and maintained on *ad libitum* water, fruit (apples, grapes, melons), vegetables (tomatoes, cucumbers), dog chow (Bonzo serial no. 651410) and chopped boiled eggs. All animals were acclimated to the laboratory for at least a week before they were used in experiments. Male laboratory mice (*M. musculus*; obtained from Harlan, USA) were maintained at the University of Wisconsin-Madison two per cage with *ad libitum* food and water. Animals (body masses and sample sizes are given in supplementary material Table S1) were killed with CO_2, and the dissected small intestines were perfused with ice-cold 0.9% NaCl.

Homogenate enzyme assay

The small intestine was cut longitudinally along its entire length into two pieces, and flash frozen in liquid nitrogen. The tissue was stored at −80°C. The combined activity of intracellular and brush-border enzymes in the small intestine was measured using a homogenate assay activity based on the glucose yield from hydrolysis (e.g. of glucosides) (Fassbinder-Orth and Karasov, 2006). For routine measures, 30 μl homogenate and 30 μl of 56 mmol l^{-1} maltose, amygdalin, lactose or sinigrin (Sigma) in 0.1 mol l^{-1} maleate and NaOH buffer, pH 6.5, were combined and incubated at 37°C for mammals and 40°C for birds for 20 min. Stop-develop reagent was added (400 μl; GAGO-20 glucose assay kit, Sigma-Aldrich), the tube was vortexed, the reaction mixture was incubated for another 30 min, and then 400 μl of 12 mol l^{-1} H_2SO_4 was added to the reaction mixture to stop the reaction. Aliquots (200 μl) were transferred to a 96-well plate, and the absorbance was read at 540 nm. For each intestinal assay, two or three replicate reactions were run, and coefficients of variation (=100×s.d./mean) averaged <10%. Each homogenate sample was corrected for absorbance in the absence of substrate hydrolysis by using a tissue blank (i.e. homogenated tissue incubated without substrate). A calibration curve of glucose concentration versus absorbance was used to determine glucose in homogenates, and hydrolysis rates are expressed as μmol min^{-1} g^{-1} wet tissue. Hydrolysis of amygdalin and sinigrin was measured at the University of Wisconsin-Madison using tissue stored for 1 year. Hydrolysis of maltose and lactose was measured at the University of Haifa/Technion, Israel and in Madison to check the effects of storage. In paired comparisons, maltase activity was 22% lower in mammalian samples measured in Madison than in Israel (t_{15}=2.4, P=0.03) but there were no significant differences in either maltase activity for birds t_{13}=0.5; P>0.6) or lactase activity for either group (for mammals, t_{15}=0.45, P>0.6; for birds, t_{13}=2.13, P>0.05).

Everted sleeve enzyme assay

This assay was used only for mice because they showed significant activity against all the glucosides. The everted sleeve assay, like the homogenate assay, is based on the rate of glucose production, expressed in μmol min^{-1} g^{-1} wet tissue. Methods from O'Connor and Diamond (1999) were followed closely.

Briefly, medial intestinal sections (where lactase activity is highest; O'Connor and Diamond, 1999) mounted on stainless steel rods were preincubated for 20 min at 37°C in stirred, oxygenated Ringer solution containing 0.5 mmol l^{-1} phlorizin at pH 7.3. Preincubation with this amount of phlorizin insures its presence in the unstirred layer adjacent to the membrane during the subsequent incubation, inhibiting 99% of mediated D-glucose uptake in mice (O'Connor and Diamond, 1999). After the initial incubation, sleeves were then incubated for 16 min at 37°C in 10 ml of oxygenated Ringer solution containing 56 mmol l^{-1} substrate (lactose or amygdalin; Sigma) and 0.5 mmol l^{-1} phlorizin at pH 6.5 stirred at 1200 rpm. After the second incubation, sleeves were taken off the rods, blotted and weighed. Two 60 μl aliquots (replicates) of the second incubation solution were added to tubes containing 400 μl of stop-develop reagent and incubated for 30 min at 37°C; 400 μl H_2SO_4 was then added to the reaction mixture and the absorbance was read at 540 nm. As described in O'Connor and Diamond (1999), we used two blanks in this assay: a reagent blank to account for impurities in the substrate solution, and a tissue blank to account for glucose leaking out of the intestinal sleeve.

Statistical data analysis

Hydrolysis of substrates by homogenates was calculated from glucose released and measured minus that in the sample's tissue blank. In figures and statistical analyses we included negative values, which can occur when glucose evolved is nil and thus the measured absorbance sometimes is less than in the blank. For each substrate–species combination, we considered substrate hydrolysis to be undetectable when the paired *t*-value, calculated as *t*=(species average)/(species s.e.m.), was not significant (P≥0.05) with N–1

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degrees of freedom (where N=number of individuals). For each substrate, comparison across species was made using one-way ANOVA followed by Bonferroni pair-wise comparisons ($P<0.05$). For these ANOVA, normal distributions were confirmed using the Komolgorov–Smirnov test, though in one case the data were $\log_{10}$-transformed to achieve this result.

For the everted sleeve samples, the hydrolysis of substrates was calculated from glucose released and measured (corrected for glucose leak from the tissue blank) versus that in the sample's reagent blank. We determined whether the substrate hydrolysis of each substrate–species combination was undetectable by methods described previously. The rates of hydrolysis of the substrates were compared between the two assay preparations (homogenate versus everted sleeve) by one-way ANOVA.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors participated in planning of the study and in elements of data collection. K.M.L. and W.H.K. did most of the data and statistical analysis and prepared the manuscript drafts, and all authors revised the manuscript. All authors were involved in developing concept and approach and in preparing and/or revising the manuscript. Experiments were performed by K.M.L., M.D.D. and W.H.K.

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Supplementary material
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