Note

Deconjugation Kinetics of Glucuronidated Phase II Flavonoid Metabolites by β-glucuronidase from Neutrophils

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Summary: Flavonoids are inactivated by phase II metabolism and occur in the body as glucuronides. Mammalian β-glucuronidase released from neutrophils at inflammatory sites may be able to deconjugate and thus activate flavonoid glucuronides. We have studied deconjugation kinetics and pH optimum for four sources of β-glucuronidase (human neutrophil, human recombinant, myeloid PLB-985 cells, *Helix pomatia*) with five flavonoid glucuronides (quercetin-3-glucuronide, quercetin-3'-glucuronide, quercetin-4'-glucuronide, quercetin-7-glucuronide, 3'-methylquercetin-3-glucuronide), 4-methylumbelliferyl-β-D-glucuronide, and para-nitrophenol-glucuronide. All substrate-enzyme combinations tested exhibited first order kinetics. The optimum pH for hydrolysis was between 3.5-5, with appreciable hydrolysis activities up to pH 5.5. At pH 4, the K_m ranged 44-fold from 22 μM for quercetin-4'-glucuronide with *Helix pomatia* β-glucuronidase, to 981 μM for para-nitrophenol-glucuronide with recombinant β-glucuronidase. V_{max} (range: 0.735-24.012 μmol$\cdot$min$^{-1}\cdot$unit^{-1} [1 unit is defined as the release of 1 μM 4-methylumbelliferyl-β-D-glucuronide per min]) and the reaction rate constants at low substrate concentrations (k) (range: 0.002-0.062 min$^{-1}\cdot$(unit/L)$^{-1}$ were similar for all substrates-enzyme combinations tested. In conclusion, we show that β-glucuronidase from four different sources, including human neutrophils, is able to deconjugate flavonoid glucuronides and non-flavonoid substrates at fairly similar kinetic rates. At inflammatory sites *in vivo* the pH, neutrophil and flavonoid glucuronide concentrations seem favorable for deconjugation. However, it remains to be confirmed whether this is actually the case.

Keywords: β-glucuronidase; deconjugation; flavonoids; quercetin; inflammation; neutrophils; enzyme kinetics; metabolism

Introduction

Epidemiological and animal experimental studies suggest that phenolic compounds such as flavonoids, present in substantial amounts in our daily diet,[1,2] beneficially affect human health.[3,4] *In vitro* studies provide a wide array of potential mechanisms through which these compounds may act.[5] Virtually all of these mechanistic studies were performed with unconjugated flavonoids, so-called aglycones. A key issue that has been largely neglected thus far, is that flavonoid aglycones do not circulate *in vivo* because of extensive phase II metabolism af-

ter ingestion.[6] In the epithelial cells of the intestinal wall and in liver, flavonoids are rapidly conjugated, primarily with glucuronic acid, catalyzed by UDP-glucuronosyltransferase.[2,7] Conjugation of bioactive compounds makes them more water soluble and consequently leads to rapid elimination of the conjugated compounds from the body via the urine. In addition to facilitating excretion, conjugation has been shown to decrease bioactivity of flavonoids in *in vitro* studies.[7,8]

Hydrolysis of the glucuronide moiety can be carried out by mammalian β-glucuronidase (EC 3.2.1.31), an enzyme present in high concentrations in the lumen of the endoplasmic reticulum and in the lysosomal fraction of cells of most tissues, but particularly the intestine and liver.[9] Deconjugation of flavonoid glucuronides could lead to prolonged circulation and enhanced bioactivity. Flavonoid glucuronides are substrates for hepatic β-glucuronidase. O'Leary *et al.*[10] showed that flavonoid glucuronides were deconjugated after entering intact HepG2 cells via an as yet unidentified transporter. Uptake of flavonoid glucuronides by cell types other than liver or intestinal cells has not been demonstrated so far and it is therefore unknown whether glucuronidated compounds reach intracellular β-glucuronidase of tissues other than the intestine and liver.

In 2000, Shimoi *et al.*[11] presented the hypothesis that β-glucuronidase released from neutrophils at inflammatory sites may be able to deconjugate flavonoid glucuronides. Lysosomal β-glucuronidase is present in high concentrations in neutrophils and eosinophils. Upon stimulation, these inflammatory cells release β-glucuronidase into the extracellular space.[12] This could lead to increased local β-glucuronidase activity at inflammatory sites and increased serum β-glucuronidase concentrations during inflammation.[13] Pre-clinical cancer studies with glucuronidated prodrugs found drug activation by β-glucuronidase excreted from neutrophils in necrotic areas of human pancreatic and lung carcinomas. This led to increased deposition of the active drug in tumor xenografts and reduced drug load to normal tissues.[14,15] β-Glucuronidase from stimulated neutrophils has the ability to hydrolyze flavonoid glucuronides *in vitro*,[13] but the deconjugation kinetics have never been studied in detail.

The work presented here describes the deconjugation kinetics of five flavonoid glucuronides, and compares them to the commercially available non-flavonoid glucuronide substrates 4-methylumbelliferyl-β-D-glucuronide (4MUglA) and para-nitrophenol-glucuronide (pNPglA). In addition, we assessed the pH optimum for β-glucuronidase, and compared the activity of human neutrophil β-glucuronidase to that of human recombinant β-glucuronidase, β-glucuronidase from the neutrophil-like tumor cell line PLB-985, and β-glucuronidase from the mollusk *Helix pomatia*.

Materials and Methods

Materials: All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise and were of analytical or HPLC grade, where applicable. Water was purified with a MilliQ system (Millipore, Watford, UK).

Enzyme sources: Four sources of β-glucuronidase were compared. *Helix pomatia* (type H5, $\geq 400,000$ units/g solid) is commonly used in sample processing to deconjugate flavonoid glucuronides present in blood plasma. *Helix pomatia* H5 is a mixture of enzymes containing β-glucuronidase and sulfatase activity.

Recombinant human β-glucuronidase isolated from murine fibroblasts was generously provided by Prof. S. R. Roffler (Institute of Biomedical Sciences, Taipei, Taiwan). The recombinant human β-glucuronidase was highly pure, active, and contained normal post-translational modifications.[16]

Finally, cell free extracts of PLB-985 cells and isolated human blood neutrophils were used as sources of β-glucuronidase. The human myeloid leukemia cell line PLB-985 can differentiate into mature neutrophils. Differentiated PLB-985 cells have been shown to closely mimic human blood neutrophils in terms of morphology, degranulation, and oxidative response.[17] Cells were cultured in RPMI-1640 culture medium with 10% fetal bovine serum (FBS; Invitrogen), 2% penicillin/streptomycin (Gibco), and 1% Glutamax (Invitrogen) in a humidified 37°C incubator with 5% CO_2. Cells were subcultured twice a week. For differentiation, cells were transferred to RPMI-1640 medium supplemented with 0.5% N,N-dimethylformamide, 0.25% FBS, 1% Nutridoma-SP (Roche), 2% penicillin/streptomycin, and 1% Glutamax, at a starting concentration of $\sim 2 \times 10^5$ cells/mL, and cultured for 6 days. Differentiation was confirmed by May-Grunwald-Giemsa staining of cytocentrifuged cells and by their ability to produce superoxide anion and release β-glucuronidase in response to stimulation with phorbol 12-myristate 13-acetate (PMA). On day 6, $\sim 1 \times 10^6$ viable cells/mL were available for experiments. Human neutrophils were isolated as described by Gungor *et al.*[18] from fresh blood of 5 healthy volunteers (4 females, 1 male; mean age 26.6 yrs, range 24–29 yrs), in accordance with standard procedures at Maastricht University. To obtain cell free extracts, PLB-985 cells were first washed with HEPES medium (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 1 mM $CaCl_2$, 5.6 mM glucose, and 0.5% human serum albumin (Sanquin, Amsterdam, Netherlands), pH 7.4), and then resuspended in reaction buffer (0.1 M sodium-acetate buffer pH 4.0; final concentration 7.2×10^6 cells/mL) containing Triton X-100 at a final concentration of 0.5%, whereas isolated neutrophils were directly resuspended in reaction buffer with Triton X-100 (final concentration
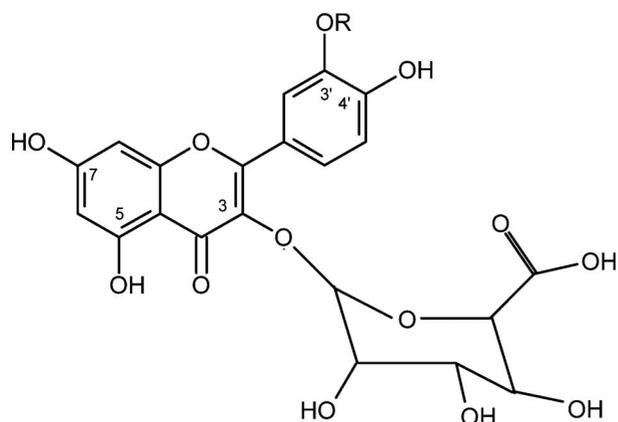
Fig. 1. Molecular structure of glucuronidated flavonoid metabolites
R=H: quercetin-3-glucuronide, R=CH$_3$: isorhamnetin-3-glucuronide.

4×10^6 cells/mL). Cells were vortex mixed and kept on ice for 10 min. Then, the samples were filtered through a 5-μm acrodisc (Pall, MI) to remove cell debris.

β-Glucuronidase activity of cell-free extracts of both PLB-985 cells and isolated neutrophils was determined on each experimental day by assessing the initial rate of hydrolysis (V$_0$) at 37°C with 100 μM 4-methylumbelliferyl-β-D-glucuronide (4MUglA) as a substrate, as described below. Similar experiments were carried out on one day in triplicate for *Helix pomatia* and recombinant human β-glucuronidase. The release of 1 μM 4-methylumbelliferyl (4MU) per min under these conditions was considered 1 unit.

Protein contents were determined in 50-μL samples by the bicinchoninic acid assay with reagents from Pierce (Rockford, IL), and bovine albumin as a standard.

Substrates: 4MUglA and pNPglA were purchased from Fluka. Quercetin-3-glucuronide (Q3glA), quercetin-3'-glucuronide (Q3'glA), quercetin-4'-glucuronide (Q4'glA), quercetin-7-glucuronide (Q7glA), and 3'-methylquercetin-3-glucuronide (isorhamnetin-3-glucuronide, IR3glA) (**Fig. 1**) were chemically synthesized as described previously.[19] Purity of the compounds was checked by HPLC with UV detection (see assay below) and was >98.5% for all compounds.

Assays for β-glucuronidase activity: To monitor hydrolysis of the glucuronide moiety, all substrates and β-glucuronidase sources were dissolved in reaction buffer (0.1 M sodium-acetate buffer pH 4.0). All assays were performed in triplicate (4MUglA and pNPglA) or duplicate (flavonoid glucuronides). Release rates of aglycones were quantified by comparison to appropriate standard curves for each substrate, and expressed as μmol·min^{-1}·mg^{-1} protein. Standard curves were all linear in the range tested (R^2>0.995). K$_m$ and apparent V$_{max}$ were calculated with the method of Lineweaver and Burk.[20] Initial rates of hydrolysis obtained at low substrate con-

centrations were not included in the Lineweaver-Burk plots if they clearly deteriorated the fit of the regression line. Eadie-Hofstee plots, which are less sensitive to variations in V$_0$ at low concentrations, were constructed to verify the results. In all cases, the Eadie-Hofstee plots gave results similar to those obtained with Lineweaver-Burk plots. The rate constant (k) of the reaction at low substrate concentrations was determined as the slope of the initial linear part of the Michaelis-Menten curve, and by calculating the ratio of V$_{max}$/K$_m$.

We used a modified 4MUglA assay to determine β-glucuronidase activity. Hydrolysis of 4MUglA was followed fluorometrically in a thermostated platereader (Spectra max m2, MDS, CA) at 37°C and 320/460 nm excitation/emission wavelengths. Usually, 4MUglA hydrolysis assays monitor fluorescence of 4MU at 360/460 nm excitation/emission wavelengths after stopping the reaction with glycine buffer pH 10.5. However, to make kinetic measurements without stopping the reaction, detection at 320/460 nm proved more sensitive. Since only the deconjugated product fluoresces, the hydrolysis can be followed continuously for 30 min without stopping the reaction. To 20 μL of β-glucuronidase solution, 40 μL of 4MUglA were added at concentrations between 0.45 and 2000 μM. The protein concentration of the β-glucuronidase solution was 14.7 mg/L for *Helix pomatia*, 1.1 mg/L for recombinant human β-glucuronidase, 160.2 mg/L for PLB-985 cell lysates, and 320.1 mg/L for the isolated human neutrophil lysates.

pNPglA deconjugation was assessed in the same platereader by absorbance measurement at 405 nm. pNPglA (40 μL) at concentrations between 7.8 and 4000 μM was incubated with 20 μL of β-glucuronidase solution at 37°C. The reaction was stopped by adding 100 μL 0.5 M glycine buffer pH 10.5 at t=0, 5, 10, 20, 30, 40, 50, and 60 min.

Hydrolysis of the flavonoid glucuronides was monitored after isocratic HPLC separation by UV detection at 375 nm for the QglA's and IR3glA. For each concentration and time point tested, 40 μL of substrate (at concentrations of 6.25, 12.5, 25, 50, 100, and 200 μM) was incubated with 20 μL of β-glucuronidase solution at 37°C. The reaction was stopped at t=0, 5, 10, 20, 30, and 40 min by adding 30 μL of ACN/phosphoric acid (80/20% v/v). Detection of the flavonoid glucuronides and aglycones was achieved by injecting 25 μL of sample onto a Chromolith RP 18e column (100 × 4.6 mm; Merck, Darmstadt, Germany) at 30°C, in an HP1100 HPLC system (Agilent, Palo Alto, CA). The eluent consisting of ACN/water/TFA (30/70/0.1% v/v/v) was run at 2.5 mL/min, and total run time for each sample was 3.5 min.

D-Saccharic acid-1,4-lactone (final concentration 4.4 mM) a β-glucuronidase inhibitor, was added to control incubations of all substrates to check whether inhibition of the enzyme completely blocked the hydrolysis of all

glucuronidated substrates.

pH optimum determination: Incubations were carried out in triplicate at different pH values to determine the effects of pH on β-glucuronidase activity with 100 μM 4MUglA and pNPglA as substrates. Instead of reaction buffer, 0.1 M sodium-acetate buffer of pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, or 0.1 M potassium-phosphate buffer of pH 6.0, 6.5, 7.0, and 7.2 was used. At each pH a standard curve of 4MU and pNP was constructed to correct for pH effects on the measurement of the reaction product. 4MU release was determined continuously for 30 min and V_0 at each pH was plotted. Release of pNP was measured at 60 min (*Helix pomatia* and human recombinant β-glucuronidase) or 120 min (cell line and isolated neutrophil β-glucuronidase). At these time points, deconjugation rates were constant. Otherwise, reaction conditions were the same as in the kinetic experiments.

Control experiments were conducted to check whether proteases possibly present in the cell-free extract of PLB-985 cells influences the β-glucuronidase activity by breaking down the enzyme. β-Glucuronidase activity was assessed in duplicate with 4MUglA as a substrate at concentrations between 3.9 and 1000 μM at pH 4 and 7.2, after preincubation of the intact cells for 10 min with 5.7 mM diisopropylfluorophosphate, and after addition of 10 μL of protease inhibitor cocktail (Sigma P8340) to 990 μL of the cell lysate.

Results

The deconjugation of five glucuronidated quercetin metabolites (Q3'glA, Q4'glA, Q3glA, Q7glA, and IR3glA) and two commercially available glucuronidated substrates (pNPglA, and 4MUglA) by β-glucuronidase from four different sources was studied to establish enzyme kinetic parameters. All substrate-enzyme combinations tested exhibited first-order kinetics.

Estimated V_{max}, K_m, and k at pH 4 for all sources and substrates tested are presented in **Table 1**. Because of the limited availability of the quercetin glucuronides, we were not able to determine hydrolysis rates above final concentrations of 140 μM, which were sometimes lower than the K_m estimated based on these experiments. This means that there is a substantial degree of uncertainty in some K_m and V_{max} values presented in **Table 1**. The hydrolyses rates at low substrate concentrations (k), also presented in **Table 1**, were not affected by this. K_m, which is independent of the amount of protein present during incubation, ranged 44-fold from 22 μM for Q4′§ glA with *Helix pomatia*, to 981 μM for pNPglA with recombinant human β-glucuronidase. The range in V_{max} and k values was much higher, > 52 000-fold and > 17 000-fold, respectively, mainly because these values are expressed relative to the total protein content, and two of the β-glucuronidase sources used were cell lysates

Table 1. Kinetic constants for the hydrolysis of flavonoid, para-nitrophenol, and 4-methylumbelliferyl glucuronides by *Helix pomatia* β-glucuronidase, recombinant human β-glucuronidase, and by β-glucuronidase from human neutrophil and PLB–985 neutrophil-like cell line lysates at pH 4

	Kinetic constants per mg or g protein			Kinetic constants per U[a]	
	V_{max} μmol·min^{-1}·mg^{-1}	K_m μM	k min^{-1}·(g/L)$^{-1}$	V_{max} μmol·min^{-1}·U^{-1}	k min^{-1}·(U/L)$^{-1}$
Helix pomatia					
pNPglA	0.274	197	1.242	2.794	0.013
4MUglA	0.097	37	2.733	0.988	0.028
Q3'glA	0.168	111	1.284	1.707	0.013
Q4'glA	0.116	22	2.969	1.185	0.030
Q3glA	0.157	183	0.831	1.598	0.008
Q7glA	0.118	129	0.848	1.206	0.009
IR3glA	0.072	71	0.898	0.735	0.009
Recombinant human					
pNPglA	7.118	981	4.962	3.269	0.002
4MUglA	4.336	207	23.723	1.991	0.011
Q3'glA	3.362	94	33.766	1.544	0.016
Q4'glA	1.854	93	14.205	0.852	0.007
Q3glA	1.880	33	66.059	0.863	0.030
Q7glA	8.356	151	57.562	3.837	0.026
IR3glA	52.283	459	104.920	24.012	0.048
PLB-985 cell line lysate					
pNPglA	0.005	695	0.006	ND	ND
4MUglA	0.002	120	0.011	ND	ND
Q3'glA	—	—	0.019	—	0.060
Q4'glA	0.005	133	0.033	5.401	0.035
Q3glA	0.001	63	0.019	2.508	0.033
Q7glA	0.006	290	0.018	3.741	0.011
IR3glA	0.011	344	0.030	6.980	0.020
Isolated human neutrophil lysate					
pNPglA	0.031	66	0.428	ND	ND
4MUglA	0.002	121	0.012	ND	ND
Q3'glA	0.007	217	0.041	4.040	0.025
Q4'glA	0.008	52	0.150	3.124	0.062
Q3glA	0.005	199	0.027	1.792	0.010
Q7glA	0.011	311	0.037	6.190	0.021
IR3glA	0.015	244	0.056	8.777	0.032

4MUglA, 4-methylumbelliferyl-β-D-glucuronide; pNPglA, para-nitrophenol-glucuronide; Q3glA, quercetin-3-glucuronide; Q3′glA, quercetin-3′-glucuronide; Q4′glA, quercetin-4′-glucuronide; Q7glA, quercetin-7-glucuronide; IR3glA, isorhamnetin-3-glucuronide; ND, not determined.
[a]One unit (U) is defined as the release of 1 μM 4-methylumbelliferyl per min.

containing little β-glucuronidase compared to total protein. The average protein concentrations present during incubation were 14.7 mg/L for *Helix pomatia*, 1.1 mg/L for recombinant human β-glucuronidase, 160.2 mg/L for PLB-985 cell lysates, and 320.1 mg/L for the isolated human neutrophil lysates. Overall, the V_{max} and k values were highest for recombinant β-glucuronidase, lower for *Helix pomatia* β-glucuronidase, and lowest but comparable for the cell lysates, reflecting the proportion of β-

glucuronidase in the total protein content. When V_{max} and k were expressed per unit (with 1 unit defined as the release of 1 μM 4MU per min), the kinetic constants for all enzyme sources were of the same order of magnitude (**Table 1**).

Compared to the other substrates tested, pNPglA had a relatively high K_m, ranking highest for 3 out of 4 enzyme sources. pNPglA combined this relatively high K_m with a relatively high V_{max} for each enzyme source. Its V_{max} ranked highest for *Helix pomatia* and isolated neutrophils, and third for the recombinant human β-glucuronidase and the human cell line, reaching its highest hydrolysis rate with recombinant human β-glucuronidase at 7.1 μmol·min^{-1}·mg^{-1} protein. V_{max} and K_m values for the four quercetin glucuronides were very similar when each source of enzyme was considered individually. The difference between the highest and lowest V_{max} ranged from 1.4- to 6-fold for the four quercetin glucuronides, whereas the highest versus lowest K_m differed 5- to 8-fold. Thus, the position of the glucuronic acid on the quercetin molecule did not seem to influence V_{max} nor the K_m considerably. V_{max} and K_m values for the hydrolysis of 4MUglA were of the same order of magnitude as those for the quercetin glucuronides. Methylation of Q3glA at the 3′-position, which yields IR3glA, enhanced the V_{max} compared to its unmethylated counterpart for human β-glucuronidase from different sources (3 to 28-fold), but decreased the V_{max} for the mollusk β-glucuronidase 2-fold. At the same time methylation resulted in a 1.2 to 14-fold higher K_m for the human β-glucuronidase sources, but a 3-fold lower K_m for the mollusk β-glucuronidase. The resulting k values for IR3glA compared to Q3glA were similar for the mollusk β-glucuronidase, but approximately 2-fold higher for all types of human β-glucuronidase.

Estimates of k values obtained by dividing the V_{max} by K_m (data not shown) were in good agreement with those obtained from the slope of the initial linear part of the Michaelis-Menten plot, provided that the slope was determined at substrate concentrations below ~10% of the K_m. On only 3 occasions k was determined at higher concentrations; in those cases the slope underestimated the calculated k by 29–43%, as expected.

The β-glucuronidase inhibitor D-saccharic acid-1,4-lactone completely blocked the production of aglycones from the glucuronides tested (data not shown). Control incubations with cell-free extract of the PLB-985 cells conducted in the presence of protease inhibitors at pH 4 and 7.2 gave results similar to those conducted without protease inhibitors. At pH 4, V_{max} was 76% with protease inhibitor cocktail and 63% with diisopropylfluorophosphate compared to no inhibitor, indicating that there was no breakdown of β-glucuronidase by proteases under the incubation conditions used, but rather that the protease inhibitors reduced enzyme activity. At
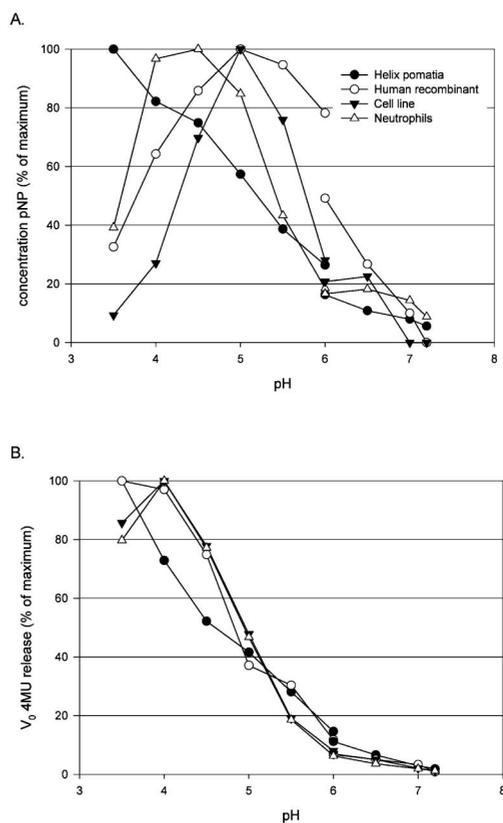

Fig. 2. Effects of pH on hydrolysis of para-nitrophenol glucuronide (A) and 4-methylumbelliferyl glucuronide (B) by *Helix pomatia* β-glucuronidase, recombinant human β-glucuronidase, and by β-glucuronidase from human neutrophil and PLB-985 neutrophil-like cell line lysates

pH 7.2, no activity was found either with or without inhibitors.

Experiments to determine the pH optimum of β-glucuronidase were carried out with 4MUglA and pNPglA. The optimum pH for hydrolysis of 4MUglA was 3.5 to 4 (**Fig. 2**), with appreciable hydrolysis activities up to a pH of 5.5. For hydrolysis of pNPglA the optimum pH seemed slightly higher and was more variable between the enzyme sources tested. For *Helix pomatia* β-glucuronidase the optimum was again 3.5, but for the other enzyme sources the optimum pH was between 4.5 and 5. At near-physiological pH 7.2, β-glucuronidase activity was low. The type of buffer used at pH 6 (0.1 M sodium-acetate or potassium-phosphate buffer) did not substantially influence activity, except for recombinant β-glucuronidase with pNP, where the activity was 29% lower when the phosphate buffer was used.

Discussion

We have shown that β-glucuronidase from four different sources has the capacity to deconjugate five flavonoid metabolites (Q3′glA, Q4′glA, Q3glA, Q7glA, and IR3glA) and two non-flavonoid glucuronidated substrates

(pNPglA and 4MUglA). The kinetics of hydrolysis of all substrates tested were remarkably similar, suggesting that β-glucuronidase has broad specificity. The different enzyme sources exhibited V_{max} and reaction rate constants k that were of the same order of magnitude for each substrate when expressed per unit enzyme activity. The differences in kinetic constants were relatively large when expressed per mg or g of protein, because the proportion of β-glucuronidase to total protein was high for the pure enzyme (recombinant human β-glucuronidase) and the purified extract (Helix pomatia), but low for the cell lysates (PLB-985 neutrophil-like myeloid cell line, and isolated human neutrophils).

Cell-free extracts from the human liver, small intestine, placenta, feces, neutrophils, and several human cell lines (liver, endothelial cells, intestine, lung fibroblast) have previously been shown to possess β-glucuronidase activity towards pNPglA and/or 4MUglA.[10,21-24] For 4MUglA, K_m values reported were 330 μM[21] for human neutrophils, and $\sim$ 50 μM for placental β-glucuronidase.[22] O'Leary et al.[24] presented kinetic constants at pH 7.2 for the hydrolysis of a mixture of several flavonoid glucuronides and pNPglA by human recombinant β-glucuronidase isolated from COS-7 cells. K_m values reported by them are of the same order of magnitude as in our study: 1400, 48, 167, and 237 μM for pNPglA, Q4'glA, Q3glA, and Q7glA, respectively, but no data were reported for Q3'glA or IRglA. Whereas in our experiments, the activity of all β-glucuronidase sources tested was low at pH 7.2, O'Leary et al.[24] reported relatively high V_{max} values at pH 7.2, of similar magnitude as we found at pH 4. In our study at pH 4 the V_{max} for human recombinant β-glucuronidase ranged from 1.9 to 8.4 μmol$\cdot$min$^{-1}\cdot$mg^{-1} protein for the three quercetin glucuronides and pNPglA, whereas in the study by O'Leary et al. at pH 7.2 the apparent activity for these compounds ranged from 0.5 to 3.4 μmol$\cdot$min$^{-1}\cdot$mg^{-1} protein,[24] only slightly lower. It is unclear why O'Leary et al. found such high activity at physiological pH.

The optimum pH for hydrolysis varied with substrate and enzyme source. For Helix pomatia the pH optimum may be even lower than the lowest value tested, which was pH 3.5. To the best of our knowledge, no data have been published on the pH optimum for Helix pomatia β-glucuronidase, although it is generally used at pH 4–5 during sample preparation for the determination of aglycone contents in plasma. For the human β-glucuronidase sources tested, we found an optimum pH between 3.5 and 5. In general, data reported in the literature agree with our values, although no identical substrate-enzyme source combinations have been tested previously. An early study on rat neutrophil β-glucuronidase reported a pH optimum of $\sim$ 5 when phenolphtalein glucuronide was used as a substrate.[25] Human recombinant β-glucuronidase had higher activity at pH 5.5 compared to pH 7 and

7.4 when N-hydroxy-diacetylbenzidine glucuronide was used as a substrate.[26] Human β-glucuronidase derived from placenta,[22] lung tumor tissue,[27] or synovial fluid[28] all exhibited optima between pH 3.5 and 5. Contractor and Shane[22] were the only ones to determine the optimal pH of 4MUglA hydrolysis by β-glucuronidase (from placenta), which they found to be 3. This agrees with the relatively low pH we found to be optimal for 4MUglA compared to pNPglA.

So far, only one β-glucuronidase isoenzyme has been identified, although differences in the degree of glycosylation that affect its activity have been observed. Both microsomal and lysosomal β-glucuronidase are derived from the same gene.[9,24] Early studies reported on a separate placental isoenzyme, with properties different from the enzyme found in plasma.[22] The placental isoenzyme was heat labile and had lower specific activity. However, these findings have not been confirmed in later studies. Next to β-glucuronidase, the mammalian enzyme UDP-glucuronyltransferase (EC 2.4.1.17) reportedly has the capacity to deconjugate glucuronic acid metabolites. However, the β-glucuronidase activity of UDP-glucuronyltransferase is UDP-dependent, and is, in contrast to the activity of β-glucuronidase, not inhibited by D-saccharic acid-1,4-lactone.[29] The β-glucuronidase activity in our experiments was completely inhibited by D-saccharic acid-1,4-lactone, suggesting that no residual UDP-glucuronyltransferase contributed to the hydrolysis rates observed. UDP-glucuronyltransferase is present in high concentrations in the liver and other metabolizing organs. If true, the β-glucuronidase activity of UDP-glucuronyltransferase may disqualify the results reported from studies with tissue cell-free extracts or microsomal fractions in which inhibition of the reaction was not checked by adding the specific β-glucuronidase inhibitor D-saccharic acid-1,4-lactone.

Several authors have shown that β-glucuronidase is remarkably stable and resistant to proteolytic breakdown. Wiener et al.[25] reported that the enzyme activity of human neutrophil β-glucuronidase lysates remained constant for up to 2 h, which was considered indicative for the absence of enzyme breakdown.[25] We found no change in reaction rates when protease inhibitors were added. This indicates that proteolytic breakdown of β-glucuronidase did not affect our data. β-Glucuronidase is also remarkably insensitive to heat and pH inactivation. Mouse kidney β-glucuronidase was stable between pH 4 and 11 for at least 30 min, and at temperatures up to 55°C for at least 10 min.[30]

Differentiated PLB-985 cells were previously shown to mimic mature neutrophils in terms of morphology, oxidative response, and functional degranulation of myeloperoxidase-negative granules and secretory vesicles.[17] β-Glucuronidase is present in the azurophil or primary granules, which are packaged with acidic hydrolases, and

are the first granules formed during granulopoiesis.[31] Our results demonstrate that β-glucuronidase from differentiated PLB-985 cells has kinetic constants for the hydrolysis of glucuronidated substrates that are similar to those obtained with isolated human neutrophils. This suggests that PLB-985 cells are a useful model system for investigating β-glucuronidase from human neutrophils.

The acute inflammatory host response is characterized by recruitment of neutrophils into inflamed tissue within minutes to hours. During subsequent migration through interstitial tissues, azurophil granules undergo partial exocytosis, resulting in the release of a host of matrix-degrading and other enzymes, including β-glucuronidase, into the extracellular space.[31] Whether β-glucuronidase enhances the bioactivity of flavonoid glucuronides at inflammatory sites will depend strongly upon local neutrophil concentrations, flavonoid glucuronide concentrations, and pH. We conducted our incubations with lysed cells at concentrations between 1.3 and 2.5×10^6 cells/mL, final flavonoid glucuronide concentrations between 4.2 and 133 μM, and optimum pH for β-glucuronidase was determined to be between pH 3.5 and 5, depending on the enzyme source and substrate. Animal models of inflammation show that neutrophil concentrations in subcutaneous pouches or sponges can by far exceed the concentrations used in our experiments, and reach up to 135×10^6 cells/mL.[25,32] However, human studies with milder inflammation have generally found lower neutrophil counts. For example, neutrophil concentrations in bronchoalveolar lavage fluid were $\sim 75 \times 10^4$ cells/mL after corn dust inhalation,[33] $\sim 12 \times 10^5$ cell/mL after LPS inhalation,[34] and up to 5.9×10^4 cells/mL in smokers.[35] Plasma concentrations of flavonoid glucuronides are typically below 1 μM.[36,37] No evidence is available so far that concentrations approaching K_m are attained anywhere in the mammalian body, except for excretory fluids like bile, in which concentrations in rats were found to approach 350 μM after intestinal perfusion with 50 μM quercetin for 30 min.[38,39] Thus, reaction rates *in vivo* will not be saturated and the deconjugation rate is best decribed by the rate constant k. This means that under the conditions used with the isolated human neutrophil lysate the half-life of quercetin glucuronides ranges from 15 (Q4'glA) to 80 (Q3glA) min at pH 4. To our knowledge, no information is available on flavonoid glucuronide concentrations at inflammatory sites. The notion of decrease in tissue pH during inflammation has been reported for different types of inflamed tissue, including atherosclerotic plaques,[40] certain solid tumors,[27] and arthritic joints,[41] as well as in animal models of subcutaneous inflammation.[32] However, accurate determination of tissue pH remains extremely difficult, and reported values vary widely. Moreover, the observed decrease in pH during inflammation is usually only modest, with lowest reported values of approxi-

mately 6.[27,32] Although we found the activity of β-glucuronidase to be much lower at higher pH values, at pH 6 approximately 10–20% of the maximum activity at pH 4, deconjugation may still occur *in vivo*. A deconjugation rate of only 10% of the maximum, would give reaction half-lifes ranging from 150 (Q4'glA) to 800 (Q3glA) min. The elimination half-life of quercetin from blood after ingestion of a single dose of quercetin glucosides is approximately 18 h,[42] suggesting that even at these lower hydrolysis rates, substantial deconjugation may occur. Thus, in vivo conditions under which β-glucuronidase from neutrophils enhance the bioactivity of flavonoid glucuronides at inflammatory sites seem achievable. However, it remains to be confirmed whether this is actually the case.

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