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A microtitre plate assay for measuring glycosidase activity

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Abstract
Glycosidases perform a wide range of functions in physiology and pathology, and are potential targets for the treatment of diseases such as influenza, cancer, AIDS and diabetes. This paper reports a convenient discontinuous colourimetric assay for the measurement of glycosidase activity. The assay utilises 4-nitrophenyl- substrates and quantities of product are determined by measuring absorbance at 405 nm. This assay is performed in a 96 well microtitre plate and has been used to characterise the properties of seven different glycosidases from bacteria, yeast and higher eukaryotes and their kinetic parameters determined. Assays in the presence of known inhibitors showed that inhibition modes can be determined, and IC_{50} and K_i values calculated. This assay appears to be of widely applicable and of general utility for the measurement of glycosidase activity and the evaluation of inhibitors.

Keywords: *Enzyme kinetics, galactosidases, glucosidases, glucuronidases, inhibitors, microtitre plate colourmetric assay, 4-Nitrophenol*

Abbreviations: *MOPS, morpholino-N-sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis*

Introduction

Sugar conjugates are ubiquitous and found within virtually all prokaryotes and eukaryotes. Sugars can be conjugated to other sugars, amino acids (especially in proteins), steroid hormones and other molecules. Examples of these conjugates include glycogen, starch and other polysaccharides, glycoproteins, natural glycosides such as salicilin and digitoxin and the glucuronate conjugates of hormones, drugs and other xenobiotics. The ubiquitous nature of these glycoside conjugates means that all organisms require enzymes for their synthesis and degradation.

Glycosidase enzymes catalyse the hydrolysis of sugar conjugates resulting in breaking of the glycosidic bond. Glycosides are ubiquitous and many glycosidases have the potential to be drug targets. Important examples of glycosidases include the influenza virus neuramidase [1], and the *Williopsis saturnus* killer toxin which has potent anti-microbial activity [2]. Glycosidases are important potential drug targets and in some cases therapies have been successfully developed. The influenza anti-viral agents Relenza™ and Tamiflu™ are inhibitors of the viral neuramidase, and have been approved for use in most of the world [1]. Increased activity of β-glucuronidase has been implicated in increased hormone levels in breast and some other cancers [3–5], and this is thought to be due to decreased excretion of the hormone by glucuronidation. Glucuronidase inhibitors, such as glucaro-1,4-lactone, have been shown to control carcinogenesis in animal models [6]. α-Glucosidases may be of value in treating diabetes and AIDS, whilst α-mannosidase inhibitors inhibit cancer metastasis [7].

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Given the importance of glycosidase enzymes, several papers have reported continuous or discontinuous colourimetric assays using chromogenic substrates [8–17]. Surprisingly, there appears to be no general evaluation and validation of these assay methods, and many of the reported methods are unsuitable for kinetic analyses of the enzymes or characterisation of inhibitors. This paper addresses this omission by studying seven different galactosidases, glucosidases and glucuronidases from bacteria, yeast, and higher eukaryotic sources.

Materials and methods

Materials

All reagents were supplied by the Sigma-Aldrich Chemical Co. or Fisher Ltd unless otherwise specified and were used without further purification. MOPS[1] buffer was supplied by BDH. 4-Nitrophenyl- substrates were supplied by Fluka. All reagents were prepared in 18 Ω Milli-Q water (Millipore). Buffer solutions were pH adjusted with NaOH or HCl using a Stuart Scientific pH meter calibrated with pH 7.0 and 4.0 or 10.0 standards (BDH).

Activity assays

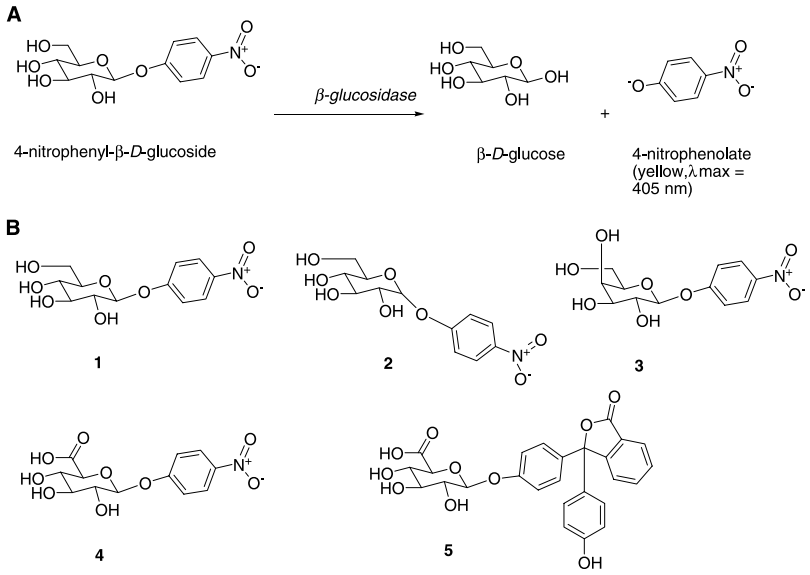
The following commercial enzymes (Sigma) were used in this study: β-*D*-Galactosidase from *Aspergillus oryzae* (G5160; E.C. 3.2.1.23); β-*D*-Galactosidase from *E. coli* (G2513; E.C. 3.2.1.23); α-*D*-glucosidase from *S. cerevisiae* (63412; E.C. 3.2.1.20); β-*D*-glucosidase from *Amygdalae dulces* (G4511; E.C. 3.2.1.21); β-*D*-Glucuronidase from bovine liver (G0251; E.C. 3.2.1.31); β-*D*-glucuronidase from *E. coli* (G7396; E.C. 3.2.1.31); and β-*D*-glucuronidase from *Patella vulgate* (keyhole limpet) (G-8132; E.C. 3.2.1.23). One unit of activity is the amount of enzyme producing 1 μmol of product per min except for the glucuronidase enzymes when one unit is the amount of enzyme producing 1 μg phenolphthalein per hour at 37°C, under the conditions defined by the Sigma-Aldrich Chemical Co. The purity of enzymes was analysed using SDS-PAGE [18] using a Mini-Protean III system (Bio-Rad) with a 3% stacking gel and 10% separating gel. Concentrated enzyme stock solutions (100 U/mL for galactosidases and glucosidases, 100 000 U/mL for glucuronidases) were made up in 20 mM buffer at their optimum pH (Table I) and stored in aliquots at −80°C.

Enzyme reaction mixtures were usually conducted in triplicate and consisted of buffer (50 mM final concentration, at the optimum pH for each enzyme), substrate (1 mM final concentration), inhibitor (if required) and water (to a final volume of 1 mL) and were pre-warmed at 37°C for 5 min. Assays were initiated using ~50 μL of enzyme in 20 mM buffer (0.2–1.5 U/mL for galactosidases, glucosidases and β-glucuronidase from *E. coli*; ~500–1000 U/mL for other glucuronidases) at 10 s intervals and 100 μL aliquots were withdrawn every 2–3 min at the same 10 s intervals for 10–15 min, to ensure that each sample was incubated for exactly the required time. The withdrawn aliquot was quenched in a 96 well microtitre plate containing 100 μL of 100 mM NaOH (assays for β-glucuronidase from *P. vulgate* required 250 mM NaOH due to the very acidic nature of the buffer) and the absorbance at 405 nm was measured using a VERSAmax tunable microtitre plate reader (Molecular Devices). Negative assay controls contained no substrate, no enzyme, or enzyme boiled at 95°C for 10 min before assaying. Control experiments showed that colour development was stable for at least 30 min after quenching with NaOH. A standard curve was used to quantify product and was made from 4-nitrophenol solution adjusted to pH 12 with NaOH (nmol product = 15.215 A_{405} + 0.8028; $R^2 = 0.9769$). The concentration of the stock solution was determined spectrophotometrically assuming $\varepsilon_{401nm} = 18.1\,mM^{-1}\,cm^{-1}$ [19]. Assays for β-glucuronidase using phenolphthalein β-glucuronide as substrate were conducted in an analogous manner except that microtitre plates were analysed at 552 nm. A standard curve of phenolphthalein was constructed assuming $\varepsilon_{552nm} = 26.6\,mM^{-1}cm^{-1}$ [20]. Protein concentrations were calculated from the U/mg data provided by the Sigma-Aldrich Chemical Co.

Table I. Assay conditions and kinetic parameters investigated for enzymes used in this study. Product was quantified by reference to a standard curve (see experimental).

	Enzyme and organism	Buffer/pH	Substrate[1]	Km (mM)	V_{max} (μmol/min/mg)
1	β-Galactosidase from *A. oryzae*	Citric acid-NaOH, pH 4.5	**3**	1.5	29.1
2	β-Galactosidase from *E. coli*	HEPES-NaOH, pH 7.3	**3**	0.16	70
3	α-glucosidase from *S. cerevisiae*	MOPS-NaOH, pH 6.8	**2**	1.3	16.8
4	β-glucosidase from *A. dulces*	Citric acid-NaOH, pH 5.0	**1**	1.8	110
5	β-Glucuronidase from bovine liver	Citric acid-NaOH, pH 5.0	**4**	0.94	0.216
6	β-glucuronidase from *E. coli*	MOPS-NaOH, pH 6.8	**4**	0.23	280
7	β-glucuronidase from *P. vulgate*	Citric acid-NaOH, pH 3.8	**4**	0.052	0.295

[1]Substrates used in kinetic assays, numbers refer to structures shown in Scheme 1.



Scheme 1. (A). Reactions catalysed by glycosidases as exemplified by β-glucosidase. Under the alkali quenching conditions 4-nitrophenol (pKa = 7.2) ionises to 4-nitrophenolate to give a yellow colour ($\varepsilon_{401nm} = 18.5\,mM^{-1}\ cm^{-1}$ [11]); (B). Structures of substrates used in this study: **1**, 4-nitrophenyl-β-*D*-glucopyranoside; **2**, 4-nitrophenyl-α-*D*-glucopyranoside; **3**, 4-nitrophenyl-β-*D*-galactopyranoside; **4**, 4-nitrophenyl-β-*D*-glucuronide; **5**, phenolphthalein-β-*D*-glucuronide.

Kinetic assays

Assays to determine kinetic parameters were conducted as for general assays except that around 8 different substrate concentrations between 0.1–10 mM final concentration were used. Most substrates are only soluble to ~40 mM in water, and hence larger volumes of stock solution were added to the assay with adjustment of the volume of water. Kinetic data was analysed by the method of Lineweaver and Burk [21] to ensure adherence to the Michaelis-Menten equation and kinetic parameters estimated using the Direct Linear Plot [22,23]. Assays containing inhibitors were performed either at fixed inhibitor concentration with variable substrate concentrations (as above) or at fixed substrate concentration (at a concentration slightly above the determined Km value) with variable inhibitor concentrations.

Results

Assays for glycosidase activity were initially conducted with the cognate substrate at a fixed concentration of 1 mM (Scheme 1). Reactions were carried out over a 10–15 minute time course with variable amounts of enzyme to determine the required quantity. In most cases this was ~50 μL of enzyme at 0.2–1.5 U/mL (galactosidases, glucosidases and *E. coli* β-glucuronidase) or 50 μL at 500–1000 U/mL (other glucuronidases) in a 1 mL assay, and all enzymes showed a linear relationship between reaction rate and amount of enzyme added. Control experiments using no enzyme or boiled enzyme showed that the substrates were stable under a wide variety of pH and buffer conditions and under the alkali quenching conditions. Thus, the coloured 4-nitrophenolate product in the assay was exclusively due to enzymatic activity. The product was quantified using a standard curve, and hence rates in units of μmol/min/mg protein can be calculated.

The substrate selectivity of each glycosidase was tested using all of the different substrates (Scheme 1) at 1 mM final concentration under the optimised assay conditions. In most cases each enzyme had almost complete selectivity for its preferred substrate. Colour development in assays with other substrates was not significantly higher than for negative controls in most cases, although weak activity was observed for a few enzyme/substrate combinations. Both the 4-nitrophenyl- and phenolphthalein- derivatives of glucuronic acid were substrates for the glucuronidases tested. The phenolphthalein derivative is a previously characterised substrate for these enzymes [20], and in some cases the rate of its conversion was approximately 10-fold higher for it compared to the 4-nitrophenyl- substrate.

Kinetic parameters for each of the enzymes were then determined. For the majority of enzymes K_m values were between 0.2 to 2 mM (Table I, **1**–**6**), but β-glucuronidase from *P. vulgate* had a lower K_m value of 0.052 mM (Table I, **7**). Values for Vmax varied more widely (~0.2–280 μmol/min/mg protein), and this is a reflection of the catalytic efficiency for conversion of the appropriate substrate. Proper Michaelis-Menten kinetics was observed for all the enzymes examined in this study over the range of substrate concentrations utilised. However, previous

experiments have shown that some enzymes exhibit non-competitive substrate inhibition in these assays (R. I. Davis and M. D. Lloyd, unpublished results), *i.e.* rates at higher substrate concentrations are lower than expected.

Experiments using known inhibitors of enzymes indicated that the expected reduction in rate was observed in their presence. Using this assay it was possible to determine IC_{50} values, *e.g.* the known suicide inhibitor conduritol-β-epoxide in our study had an IC_{50} value of 0.57 mM for *A. dulces* β-glucosidase. The reported K_i value for conduritol-β-epoxide and human β-glucosidase is 166 ± 57 μM [24]. It was also possible to determine inhibition mode and K_i values, *e.g.* in our study glucuronic acid and gluconolactone were both competitive inhibitors of β-glucuronidase from *E. coli*, with K_i values of 0.60 mM and 0.49 mM, respectively.

Discussion

Nitrophenyl-glycoside conjugates of sugars were chosen as substrates as they are commercially available, for their stability and the stability of the coloured nitrophenol product under the assay conditions. Our assays used 4-nitrophenyl- conjugates as the extinction coefficient of the 4-nitrophenolate product ($18.1\ mM^{-1}\ cm^{-1}$ [19]) gives the greatest sensitivity. The assay methods are also applicable to 2-nitrophenol derivatives, but their use will result in lower sensitivity due to the lower extinction coefficient of 2-nitrophenolate ($3.5\ mM^{-1}\ cm^{-1}$ [25]). The 4-nitrophenyl-glycoside conjugates were substrates for all of the enzymes tested in our study, even those enzymes (β-glucuronidases) traditionally assayed using alternative substrates (phenolphthalein-glucuronide). Phenolphthalein-glucuronide is considerably more expensive than the 4-nitrophenol derivative and colour development in our phenolphthalein-based assays was observed to be unreproducible under our assay conditions, possibly due to tautomerisation of the phenolphthalein product [26]. Thus, 4-nitrophenyl-glucuronide is the first choice as substrate if it is converted by the enzyme under study.

Many of the previously reported assays [8,11,15,16,25] have utilised neutral pH values (~7.0), and did not adjust the pH before the quantifying product. Since the pKa of 4-nitrophenol/4-nitrophenolate is reported to be 7.14 [19] and the absorbance of 4-nitrophenol is weak ($0.2\ mM^{-1}\ cm^{-1}$ [19]), this will result in serious under-estimation of the amount of product, assuming no change in the product pKa under the assay conditions. Moreover, this error is likely to be highly variable at neutral pH values, since small changes in pH will lead to relatively large changes in the proportion of the 4-nitrophenolate anion. At acidic pH values the assay would be extremely insensitive because the measured product is 4-nitrophenol not 4-nitrophenolate. To overcome these problems we used a discontinuous assay and quenched under alkali conditions (pH > 12). Our procedure both stops the reaction and ensures full colour development allowing proper product quantification. The procedure worked even for enzymes assayed under very acidic conditions (*e.g.* β-glucuronidase from *P. vulgate*, pH 3.8), which could not be assayed using a continuous method. A similar procedure to ours using a 96 well plate [17] has been reported, but this assay was not utilised for kinetic analyses of enzymes.

Unlike previous studies [11], our study shows that this type of assay is applicable to enzymes utilising different glycoside moieties and to enzymes from a variety of prokaryotic and eukaryotic sources. Kinetic parameters could be obtained for all of the enzymes (Table I), but it was necessary to rigorously characterise each enzyme/substrate pair in order to identify appropriate assay conditions. When screening for competitive and mixed inhibitors at fixed substrate concentrations (*i.e.* to determine IC_{50} values) a similar or slightly higher substrate concentration to their *K*m value is appropriate. This is because the characteristic of competitive inhibition is that the *K*m value apparently increases with consequent decreases in rate and high substrate concentrations will negate this effect. Moreover, non-competitive substrate inhibition is more likely to be observed at high substrate concentrations, thereby increasing the complexity of the analysis. Using known inhibitors of our enzymes we demonstrated that IC_{50} values, inhibition mode and K_i values can be determined using our assay. Thus, our assays seem to be widely applicable to the measurement of glycosidase enzyme activity, screening for inhibitors and the determination of kinetic and inhibition constants. The current assay system could be adapted to a 96 well plate format relatively easily for the medium throughput determination of IC_{50} values, although the concentrations of enzyme and substrate will need to be individually optimised.

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