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The method of integrated kinetics and its applicability to the *exo*-glycosidase-catalyzed hydrolyses of *p*-nitrophenyl glycosides

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ABSTRACT

In the present work we suggest an efficient method, using the whole time course of the reaction, whereby parameters k_{cat}, K_m and product K_I for the hydrolysis of a *p*-nitrophenyl glycoside by an *exo*-acting glycoside hydrolase can be estimated in a single experiment. Its applicability was demonstrated for three retaining *exo*-glycoside hydrolases, β-xylosidase from Aspergillus awamori, β-galactosidase from *Penicillium* sp. and α-galactosidase from *Thermotoga maritima* (TmGalA). During the analysis of the reaction course catalyzed by the TmGalA enzyme we had observed that a non-enzymatic process, mutarotation of the liberated α-D-galactose, affected the reaction significantly.

1. Introduction

By 'integrated kinetics' we mean use of the Michaelis–Menten equation in its integrated form. First described by Schwert,[1] its successful application to the lactate dehydrogenase-catalyzed reduction of pyruvate was published in 1969.[2] Despite the theory being summarized in a classic text,[3] it has not become widely used, and most enzymologists prefer to use initial velocities for estimating kinetic parameters. This method, described by Michaelis a century ago[4] is convenient because it allows product accumulation and substrate consumption to be ignored. Indeed, in the initial stages of enzyme kinetic studies, the main task is to understand the form of the kinetic law, and to locate the ranges within which kinetic parameters lie, ignoring product accumulation and substrate depletion in the first instance. Nevertheless, quite often accumulation of the product(s) and depletion of the substrate(s) cannot be neglected.

Traditionally, in glycosidase assays, model substrates are used, in which the glycone is a monosaccharide and the aglycone is a chromogenic or a fluorogenic group. Kinetic constants can then be easily determined from initial velocities measured by absorbance or fluorescence. Where estimation of initial rates is difficult (e.g., because a few percent reaction at substrate concentrations at or below the K_m gives very small absorbance changes), kinetic parameters can be more reliably derived from analysis of a whole kinetic curve. At the same time, the method of integrated kinetics enables determination of all the constants needed to characterize a reaction with a relatively small set of experiments. Using the integral form of the Michaelis–Menten equation, one can determine inhibition type, the corresponding parameters K_m, K_I, k_{cat} by varying initial substrate concentrations.

Here, we suggest a method of integrated kinetics to estimate parameters k_{cat}, K_m and K_I for the hydrolysis of a *p*-nitrophenyl glycoside by an *exo*-acting glycoside hydrolase in a single experiment, and demonstrate its applicability for three enzymes, β-xylosidase from Aspergillus awamori (GH3; (CAZy; http://www.

Abbreviations: TmGalA, α-galactosidase from *Thermotoga maritima*; *p*NP, *para*-nitrophenyl; *p*NPαGal, *p*NP-α-D-galactopyranoside; *p*NPXyl, *p*-nitrophenyl β-xylopyranoside; *p*NPβGal, *p*-nitrophenyl β-galacopyranoside.

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cazy.org),[5,6] β-galactosidase from *Penicillium* sp. (GH35)[7] and α-galactosidase from *Thermotoga maritima* (GH36).[8,9] During the analysis of the reaction course catalyzed by the α-galactosidase from *Thermotoga maritima* (TmGalA) we had observed that a non-enzymatic process, mutarotation of the liberated α-D-galactose, affected the derived parameters. This effect was reported previously for the β-galactosidase-catalyzed hydrolysis of lactose[10–12] but, to our knowledge, never for α-galactosidases. In the present work, a kinetic model invoking simultaneous competitive inhibition of the *exo*-glycoside hydrolase by the reaction product and by its spontaneously-formed anomers, with different K_I values, is suggested for the first time.

2. Results and discussion

One of the possible mechanisms for enzymatic hydrolysis can be described with Scheme 1, when competitive inhibition takes place.

The reaction includes formation of the Michaelis complex *ES* followed by release of the product *P* and regeneration of the enzyme *E*. In this case the hydrolysis rate is described by the well-known Michaelis–Menten equation:[4]

$$V = \frac{k_{cat}E_0S}{K_m + S} \tag{1}$$

where V is a reaction velocity, S, E_0 are corresponding initial concentrations of a substrate and enzyme, k_{cat}, K_m are kinetic parameters of the reaction: the graph describes a rectangular hyperbola The Michaelis–Menten Eq. 1 can be integrated[1] and written as Eq. 2:

$$k_{cat}E_0t = S - S_0 + K_m \ln\left(\frac{S_0}{S}\right) \tag{2}$$

The Eqs. 1 and 2 are valid under steady-state conditions, when the flux between all forms of the enzyme is much less than the flux between the product and the substrate. For the method we used in the present work one needs to consider two issues. First, in case of a competitive inhibition at fixed inhibitor concentrations, a form of the Eqs. 1 or 2 remains unchanged while K_m is increased by a factor of $(1+I/K_I)$ and k_{cat} is not altered.[3] So, Eq. 2 can be rewritten as:

$$k_{cat}^{app}E_0t = S - S_0 + K_m^{app} \ln\left(\frac{S_0}{S}\right) \tag{3}$$

where K_m^{app} and k_{cat}^{app} are apparent kinetic parameters that relate to true K_m and k_{cat} by equations

$$K_m^{app} = K_m \times (1 + I/K_I), \quad k_{cat}^{app} = k_{cat}.$$

From this, analyzing dependency K_m^{app} on inhibitor concentration, one can derive K_I and K_m, traditionally in form of a Dixon plot.[13]

$$E + S \underset{k_-}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$E + I \overset{K}{\rightleftharpoons} EI$$

Scheme 1. Minimal kinetic scheme for hydrolysis reaction with competitive inhibition, following Michaelis–Menten mechanism.

In case of the inhibition by a product, the concentration of which is changing during the reaction course, the expression for the reaction velocity can be written using equation of a material balance $S_0=S+P$ and assuming that $S_0>>E_0$:

$$V_{IP} = \frac{k_{cat}E_0S}{S + K_m\left(1 + \frac{S_0 - S}{K_I}\right)} \tag{4}$$

After simple transformations the Eq. 4 may be rewritten as

$$V_{IP} = \frac{k_{cat}^{app}E_0S}{S + K_m^{app}} \tag{5}$$

where

$$K_m^{app} = \frac{K_m + S_0\frac{K_m}{K_I}}{1 - \frac{K_m}{K_I}} \tag{5a}$$

and

$$k_{cat}^{app} = \frac{k_{cat}}{1 - \frac{K_m}{K_I}}. \tag{5b}$$

The equations 5aand 5b are valid for the case $K_m \neq K_I$, otherwise a dominator is zero and the whole expression becomes undetermined. However, in the case $K_m=K_I$, the velocity V can be expressed as $K\times S$ where K is the first order rate constant equal $k_{cat}/(K_m+S_0)$. One can easily distinguish this particular case from the first order reaction due to dependence of K on the initial substrate concentration S_0. Eq. 3 is well known[3] though, as a rule, it has been expressed in another form. In general, at a single initial substrate concentration, Eq. 3 makes it impossible to distinguish the effect of product inhibition from any other retarding process, whereas varying initial concentrations of the substrate enables such discrimination (a review of analytic methods of time-course of enzyme reactions are given by Cornish-Bowden[3]). Here, we suggest a convenient and clear method to determine kinetic parameters based on Eq. 5a. Indeed, integration of Eq. 5 leads to Eq. 3; values for K_m^{app} and k_{cat}^{app} may be easily obtained by non-linear fitting of the whole kinetic curve. Finally, from the dependence of K_m^{app} on the initial concentration of the substrate S_0 (Eq. 5a) one can derive two parameters, K_m and K_I.

It is clear that K_m^{app} is a linear function of the initial substrate concentration S_0 with a slope $K_m/K_I - K_m$ and y-intercept $K_m^{app}(S_0) = K_m/1 - \frac{K_m}{K_I}$. Therefore, the linear dependence of K_m^{app} on S_o provides a convenient method of analysis. A set of time courses at various values of S_o and their fitting to Eq. 3 will result in a set of K_m^{app} and k_{cat}^{app} values. Linear regression of K_m^{app} as a function of S_o yields two parameters, the slope A and the y-intercept B. Using Eq. 5a one can derive kinetic constants $K_m = B/1 + A$ and $K_I = B/A$ and the catalytic constant $k_{cat} = k_{cat}^{app}/1 + A$ from Eq. 5b. So, this approach enables one to determine k_{cat}, K_m and K_I in a simple experiment with variation of S_0.

To test the methodology, we used enzymes available in our laboratory, the β-xylosidase from *A. awamori*,[5,6] the β-galactosidase from *Penicillium* sp.[7] and the α-galactosidase from *Thermotoga maritima*.[8,9] Our aim was to analyze a dependence $K_m^{app}(S_0)$ for every enzyme and compare values for kinetic parameters obtained by two methods: the widely used analysis of the initial velocities and the method of the integral kinetics (eq. 5a). All three enzymes produce the aldopyranose product in the same anomeric configuration as the substrate. All enzymes probably act as *exo*-glycosidases with only one glycone-binding subsite (i.e., the −1

subsite)[6,7,9] and the monosaccharide product of the hydrolysis of *p*NP glycoside by each of these enzymes may very well be a competitive inhibitor for the corresponding reaction.

For the β-D-xylosidase and β-D-galactosidase kinetic properties obtained by the method of initial velocities revealed xylose and galactose were competitive inhibitors. Kinetic parameters obtained by the two methods were generally comparable (Table 1). But what was more important, dependences $K_m^{app}(S_0)$ were linear in both cases and showed good reproducibility, with an error bar of less than 2% (an example of such linear dependence for the β-galactosidase is given in Fig. 1). At this stage the method seemed generally successful.

In the case of TmGalA, we expected to see a similar linearity for the $K_m^{app}(S_0)$ dependence. However, we observed variations in the obtained values for K_m^{app} in the range of high initial substrate concentrations (5 mM and higher) at different enzyme concentrations (Fig. 2): a threefold variation in TmGalA concentrations resulted in differences between K_I values obtained from Eq. 5a of 40%. Besides the approaches used for the analysis of the data, the only difference for each experiment was the length of time period, for which the reaction was run. Our assumption was that the disagreement between K_I values may be caused by another process accompanying the enzymatic reaction. We supposed that the mutarotation of α-D-galactopyranose released by the enzyme during the hydrolytic reaction affects the kinetic parameters in the reaction course, and that the new forms generated by mutarotation (largely the β-pyranose form) had K_I values different from α-D-galactopyranose. A similar effect was reported for the β-galactosidase from *Aspergillus niger* that distinguished between α- and β-galactose in the hydrolysis of lactose.[10] Analysis of the X-ray structures of α-galactosidases belonging to different GH families suggested different affinities of these enzymes to α- and β-forms of D-galactopyranose. Thus, the crystal structure of the α-galactosidase from *Lactobacillus acidophilus* (36 GH[14]) revealed α-D-galactopyranose in the active center, whereas the crystal structure of the 27 GH family α-galactosidase from *Trichoderma reesei* contained β-D-galactopyranose.[15]

To illustrate binding variants of α- and β-galactopyranose in the active center of TmGalA, they were assessed with flexible molecular docking. Four different protonation states of the pair of catalytic residues Asp327 (nucleophile) and Asp387 (acid/base) were examined including: Asp327/Asp387, Ash327/Asp387, Asp327/Ash387 and Ash327/Ash387 (Asp is charged aspartic acid, Ash is neutral protonated aspartic acid). Binding propensities of the two galactopyranose anomers were evaluated from calculating binding scores. Results of the calculations are given in Supplemental data.

The interconversion of the α- and β-anomeric forms of D-galactopyranose occurs as the sugar ring opens to the *aldehydo* form and recloses, yielding the opposite anomeric configuration. Besides both α- and β-galactopyranose, and traces of an acyclic form, two anomeric furanoses, α- and β-galactofuranose, are involved in the mutarotation. This process is catalyzed by general acids and bases.[16] Accurate modeling of galactose mutarotation is quite complicated, therefore several assumptions were made to simplify the kinetic model. First, we assumed furanose forms can be neglected due to their presence in the amount of 4.9% (determined by NMR under conditions of the kinetic experiments) of the total amount of galactose and therefore the mixture consists generally of galactopyranoses. Since the mixture of α- and β-anomers of galactopyranose at the equilibrium consists approximately of 33.3% α-form and 61.8% β-form at 37 °C, these values were used for calculations. Our data generally agree with those previously published,[17] which pertain to slightly different conditions (100% of D_2O at 31 °C). Second assumption was that furanoses would not influence TmGalA kinetics. The ring closure to five membered rings is faster than the closure to six-membered rings, so transient accumulation of furanose forms could affect results, especially if the furanose

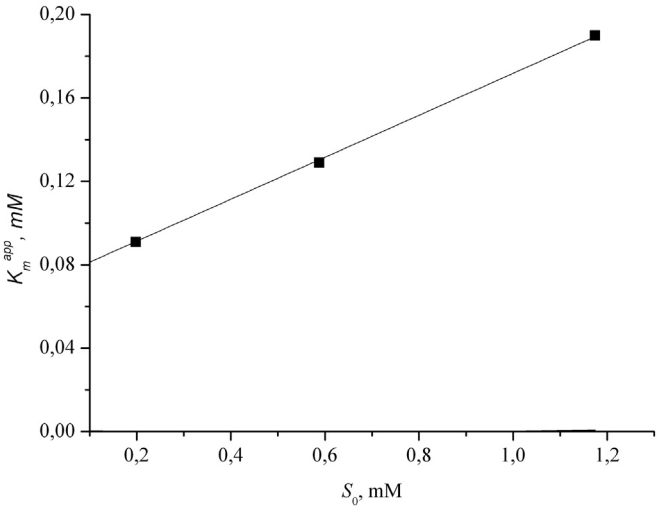


Fig. 1. K_m^{app} dependence on S_0 concentration in *p*NPβGal hydrolysis catalyzed by the β-galactosidase from *Penicillium* sp.

Table 1
Kinetic parameters for the hydrolysis of the *p*NP glycoside by β-D-xylosidase and β-D-galactosidase

Kinetic parameters	Method of initial velocities	Method of integrated kinetics
β-D-xylosidase[a]		
K_m, mM	0.28±0.03	0.29±0.015
k_{cat}, s^{-1}	65±4	71±3
K_I, mM	1.06±0.09	0.95±0.06
β-D-galactosidase[b]		
K_m, mM	0.30±0.01	0.37±0.04
k_{cat}, s^{-1}	54±2	40±2
K_I, mM	0.91±0.03	1.25±0.07

[a] Experiments were carried out at pH 4.5 and 37 °C, see details in sections 3.2 and 3.3 of Experimental.
[b] Experiments were carried out at pH 5.0 and 37 °C, see details in sections 3.2 and 3.3 of Experimental.

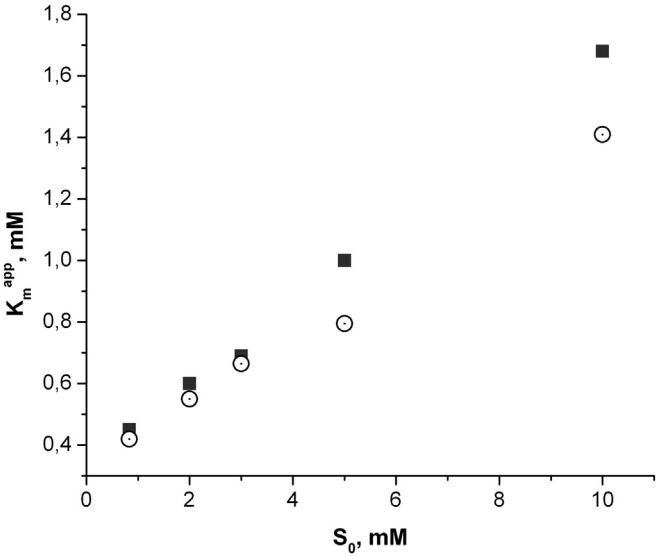


Fig. 2. K_m^{app} **dependences on** S_0 **concentration in** *p***NPαGal hydrolysis catalyzed by TmGalA**: ■ is the enzyme concentration E_0=0.25 μM; ○ is E_0=0.75 μM.

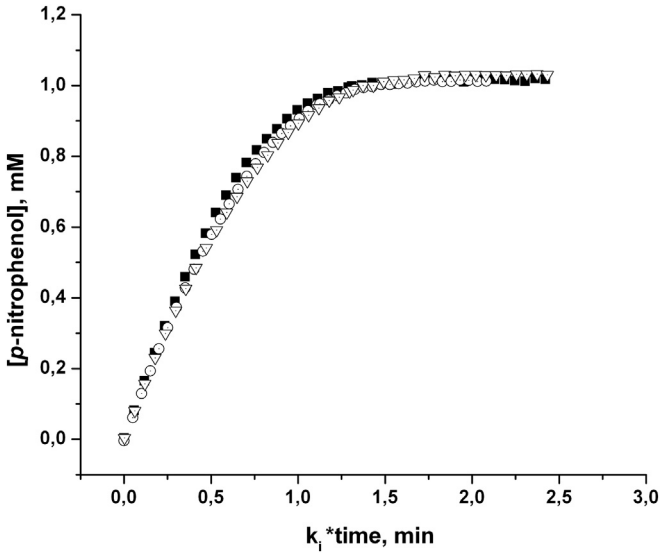


Fig. 3. Normalized enzyme kinetic curves for *p*NPαGal hydrolysis by TmGalA. Initial substrate concentration was 1 mM; (each time point is multiplied by ratio of enzyme concentrations $k_i=E_i/E_1$): ○ is E_1=0.17 μM, ■ is E_2=0.08 μM, ∇ is E_3=0.04 μM.

forms bound more tightly than pyranose forms. However, the equilibrium between α-D-pyranose and all furanose forms is reached faster than the equilibrium between α- and β-pyranoses.[18] Therefore, the ratio of α-D-pyranose to furanoses is kept unchanged during the whole process and binding of the TmGalA with any of the furanose forms is proportional to α-D-galactopyranose concentration and is indistinguishable in a kinetic experiment. Measurement of the equilibrium of tautomers under the kinetic experiments (50 mM NaAc, pH 5.0) showed that at temperature lowering, the equilibrium between tautomeric forms is changing: at 25 °C the amount of α*p* was 33%, β*p* was 62.14%, α*f* was 2.04%, β*f* was 2.84%; at 4°C: α*p* was 26.04%, β*p* was 72.0%, α*f* was 0.81%, β*f* was 1.1%. As the temperature is lowered from 25 °C to 4 °C the ratio of furanose forms to α-D-pyranose decreases twofold, from 0.148 to 0.073 However, at 4 °C K_I is approx. the same as at 37 °C and thus, binding with furanose does not, probably, affect the inhibition due to noticeable decrease of concentration of furanose forms.

To demonstrate the effect of spontaneous mutarotation of the product on the time-courses of TmGalA-hydrolysis of *p*NPαGal, we altered the enzyme concentration so that a high conversion of substrate was attained over widely differing time intervals. When the reaction time did not exceed 5 min (achieved by the use of stopped flow equipment), the time-courses were superimposable when the time axis was normalized by the enzyme concentration (i.e., one set of curves with an enzyme concentration E_o and t_{obs} as the *x* axis, another set of curves with $2\times E_o$ and $2\times t_{obs}$ as the *x* axis) by time proportional to enzyme concentration (Fig. 3). It means that mutarotation does not affect the total reaction rate and only enzymatic hydrolysis and any inhibition by α-galactopyranose takes place. Otherwise, under long-term reaction conditions (more than 15 min) normalized enzyme kinetic curves were not imposed (Fig. 4B).

The experiment with various concentrations of the enzyme enabled us to assess the inhibitory power of α- or β-galactose anomers. At the highest TmGalA concentration, the flux of the enzymatic reaction far exceeded the flux from α- to β-galactose from spontaneous reaction, and α-galactose remained predominant in the reaction mixture. Conversely, the equilibrated mixture of α- and β-anomers of galactose (33.3 and 61.8%, respectively) accumulated at the smallest enzyme concentration when the enzymic flux was less than the mutarotation flux. Therefore if one superimposes such normalized curves, the curves diverge. In the first case, the reaction rate did not depend on either the enzyme or α- and β-galactose concentration in contrast to the second case. The slower reaction in normalized coordinates corresponds to stronger inhibition and it would mean the higher affinity of α-anomer (lower value for K_I). Fig. 5B shows this: apparent acceleration at around 50% reaction can only be brought about in an enzyme we have shown to be Michaelian by spontaneous removal of an inhibitor to a less inhibitory species, in this case β-galactopyranose. Therefore we can conclude that in cases of the fastest and slowest reactions inhibition occurs by α-anomer and by the equilibrated

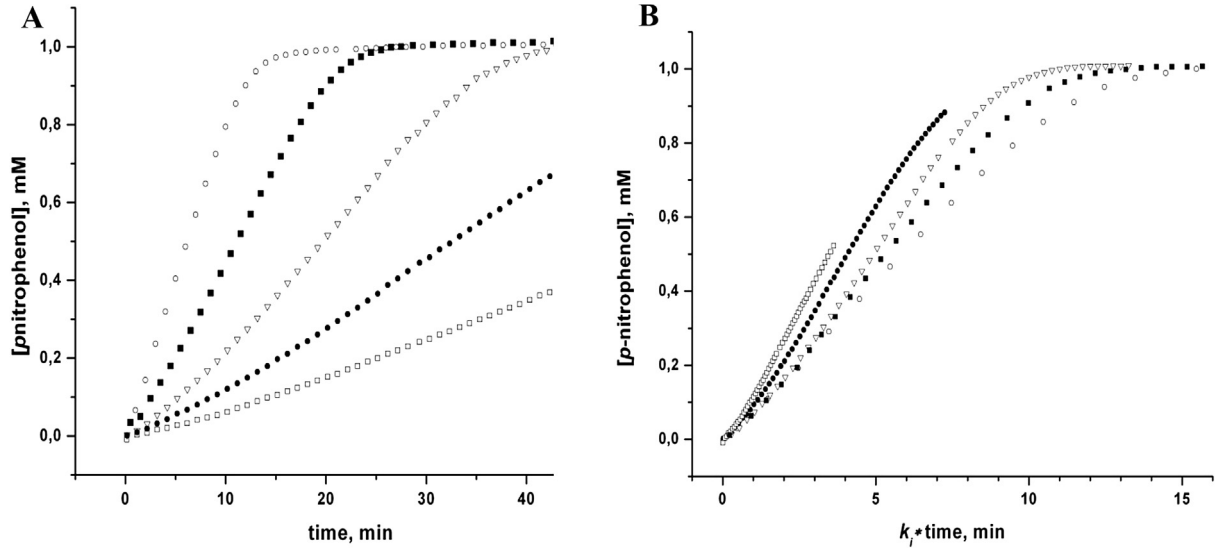


Fig. 4. Effect of D-galactose interconversion in the hydrolysys of *p*NPαGal by TmGalA. A: Enzyme kinetics for *p*NPαGal hydrolysis by TmGalA with fixed initial substrate concentration at 1 mM, reaction temperature was 37 °C. B: Normalized enzyme kinetic curves from plot *A* (each time point is multiplied by ratio of enzyme concentrations $k_i=E_i/E_1$). For both plots ○: E_1=0.55 μM, ■: E_2=0.28 μM ∇: E_3=0.14 μM, ●: E_4=0.07 μM, □: E_5=0.03 μM.

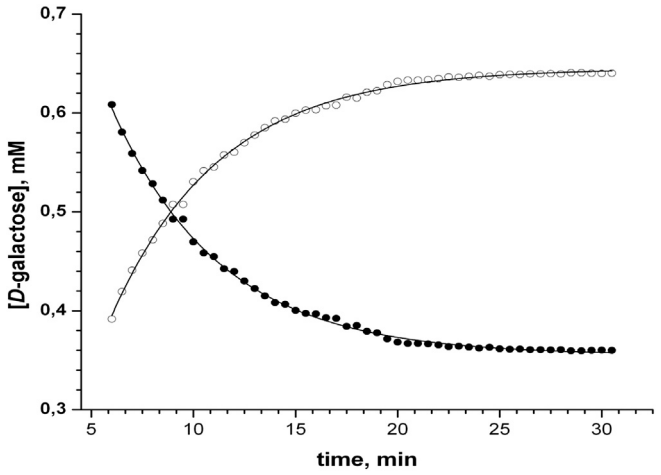


Fig. 5. Kinetics of D-galactose mutarotation monitored by NMR in situ at 37 °C. For α-D-galactopyranose—●, for β-D-galactopyranose—○.

mixture (α-+β-), respectively. When the reducing sugar product is fully equilibrated, the rate of mutarotation does not influence the overall reaction. However, one should include mutarotation into the minimal kinetic scheme of hydrolysis when comparable rates of the enzymatic reaction and mutarotation (under certain temperature and pH) take place.

Since D-galactose is a competitive inhibitor of TmGalA hydrolytic reaction and mutaroтaion is pH- and temperature-dependent, we studied the kinetics of α-/β-galactose interconversions under defined conditions by NMR spectroscopy, yielding mutarotation rates: $k_{\alpha\leftrightarrow\beta}$=0.002 s^{-1} $k_{\beta\leftrightarrow\alpha}$=0.001 s^{-1}. Kinetic data (Fig. 5) shows that the balance of α- and β-galactose can be reached within 25 min at 37 °C. Lowering the temperature to 4 °C led to the decrease of mutarotation rates approximately 50 times ($k_{\alpha\leftrightarrow\beta}$=0.000045 s^{-1} $k_{\beta\leftrightarrow\alpha}$=0.000016 s^{-1}). Thus, we considered the mutarotation to be negligible at 4 °C for 30 min, since the β-galactose percentage does not reach 7.5% of total galactose. A similar temperature dependence was observed for galactose mutarotation by Fleschel et al.[10] Thus, incubation at 4 °C made it possible to distinguish between inhibition by α- and β-forms of D-galactose in case of TmGalA-catalyzed hydrolysis of *p*NPαGal. Mannose mutarotation during *Cellulomonas fimi* β-mannanase hydrolysis was studied similarly.[19]

Further experiments with two types of inhibitors were carried out on ice. Inhibitors were: a). α-D-galactopyranose obtained by fast dissolution of crystalline α-D-galactose in water immediately before the experiment, and b). an equilibrated solution of the α- and β-D-galactoses. The values for K_I^{α} and $K_I^{\alpha+\beta}$ were calculated and are shown in the Table 2. Value for K_I^{α} in inhibition studies with equilibrated mixture of α- and β-anomers was calculated in the assumption that the equilibrated concentration of α-D-galactose is 26.5% of a total initial one. Value for K_I^{β} was calculated according to the Eq. 7, derived from Eq. 6 valid for the inhibition experiment with equilibrated mixture of α- and β-D-galactose.

Table 2
Inhibition constants for anomers of the D-galactopyranose in the hydrolysis of *p*NPαGal

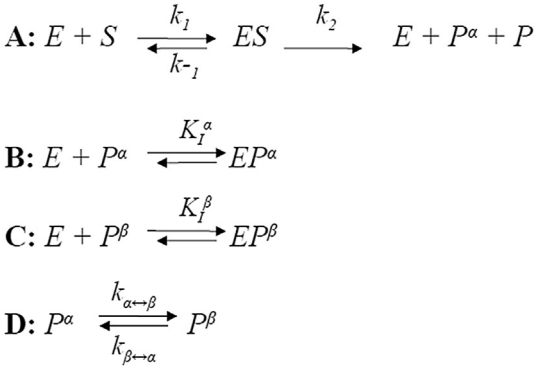
	K_m^{app}, mM	$K_I^{\alpha+\beta}$, mM	K_I^{α}, mM	K_I^{β}, mM
No inhibitor	0.070±0.005			
α-galactose	0.32±0.04		0.28±0.03	
(α+β)-galactose	0.16±0.01	0.78±0.05	0.28±0.03	2.2±0.5

Reactions were carried out for 20 min, at 4 °C.

$$\frac{I}{K_I} = \frac{I_\alpha}{K_I^\alpha} + \frac{I_\beta}{K_I^\beta}; \qquad K_m^{app} = K_m\left(1 + \frac{I_\alpha}{K_I^\alpha} + \frac{I_\beta}{K_I^\beta}\right) \tag{6}$$

$$K_I^\beta = \frac{I_\alpha \cdot K_I^\alpha \cdot K_I}{K_I^\alpha - I_\alpha \cdot K_I}; \qquad I_\alpha = 0.265I; \qquad I_\beta = 0.735I \tag{7}$$

Finally, we compared kinetic parameters obtained previously[9] with results of calculations derived from the integrated rate Eqs. 5a and 5b, considering galactose interconversion. The minimal kinetic scheme describing the *p*NPαGal hydrolysis by TmGalA is presented in Scheme 2.



Scheme 2.

where *S* is *p*NPαGal; P^{α} is α-D-galactose; P^{β} is β-D-galactose; *P* is *p*-nitrophenol.

For the scheme of the hydrolysis without inhibition, two independent catalytic constants have to be determined, i.e., k_{cat} and K_m for the *p*NPαGal hydrolysis (equation **A**). When competitive inhibition is considered, equations **A** and **B** are valid for fast or slow reactions. For the fastest reactions inhibition is determined by the α-anomer, in the slowest ones the equilibrated mixture (α-+β-) inhibits the reaction. When comparable rates of the enzymatic reaction and mutarotation take place, equations **C** and **D** should be added into the minimal kinetic scheme of the hydrolysis.

Data from the Table 3 demonstrate that the K_I value obtained using the integrated form of Michaelis kinetics differs from those determined by the standard Dixon method, because normally one uses approximately equilibrated mixture of α- and β-galactose in inhibition studies.

In conclusion, the method of integrated kinetics used in the present work can be easily applied for analysis of the glycoside hydrolase-catalyzed hydrolysis of *p*NP glycosides. However, where there is product inhibition, and mutarotation takes place on a timescale comparable to the period for which the reactions are followed, more complex treatments must be applied, particularly, as with TmGalA, where the first-formed anomer binds more tightly than the products of its mutarotation, yet these dominate the mutarotational equilibrium. Thus, a more complicated reaction model including mutarotation is required for determination of kinetic parameters of the hydrolysis.

Table 3
Kinetic parameters of *p*NPαGal hydrolysis obtained by methods of the initial velocities and integrated Michaelis–Menten kinetics[a]

Kinetic parameters	Michaelis–Menten-type kinetics	Method of the integrated kinetics (not considering mutarotation)[b]	Method of the integrated kinetics (considering mutarotation)
K_M, mM	0.080±0.005[c]	0.060±0.002	0.060±0.002
k_{cat}, s^{-1}	8.6±0.6[c]	9.1±0.3	9.1±0.3
K_I, mM	0.80±0.06[d]	0.63±0.02	K_I^{α}=0.32±0.02 mM; K_I^{β}=5.7±0.1 mM

[a] Experiments were carried out at pH 5.0 and 37 °C.
[b] The reaction was carried out for 30 min that corresponded approx. 90% conversion of the substrate.
[c] Data from the Ref. 9.
[d] Data obtained by Dixon method.[13]

3. Experimental

3.1. Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, USA) or Acros Organics (Geel, Belgium) unless otherwise noted, and were used without further purification. Substrates *p*-nitrophenyl β-D-galactopyranoside and *p*-nitrophenyl β-D-xylopyranoside were synthesized from the corresponding monosaccharides according to the method described for β-glucosides;[20] *p*-nitrophenyl α-D-galactopyranoside (*p*NPαGal) was synthesized from D-galactose as previously described for α-glucoside.[21]

3.2. Enzymes and enzymes assays

The β-D-xylosidase from Aspergillus awamori X-100 was purified as described previously.[5,6] The β-D-galactosidase from *Penicillium* sp. was purified according to the procedure reported in Ref. 7 A plasmid containing the α-D-galactosidase gene was kindly donated by Prof. R.M. Kelly (North Carolina State University, USA). The α-D-galactosidase gene from *Thermotoga maritima* MSB8 was expressed in *E. coli* BL-21(DE3) and the recombinant enzyme was purified from cells grown overnight as previously described.[9]

β-Xylosidase activity towards *p*-nitrophenyl β-D-xylopyranoside (*p*NPXyl) was determined at 37 °C in 50 mM sodium acetate buffer, pH 4.5. One unit of the β-D-xylosidase activity was defined as an amount of the enzyme releasing 1 μM of *p*-nitrophenol from *p*NPXyl per min.

One unit of the β-D-galactosidase activity towards *p*-nitrophenyl β-D-galactopyranoside (*p*NPβ Gal) was defined as the amount of the enzyme required to hydrolyze 1 μM of the substrate per 1 min at 37 °C in 50 mM sodium acetate buffer, pH 5.0.

The α-D-galactosidase activity towards *p*-nitrophenyl α-D-galactopyranoside (*p*NPαGal) was determined at 37 °C in 50 mM sodium acetate buffer, pH 5.0. One unit of the activity was defined as amount of the enzyme releasing 1 μM of nitrophenol from *p*NPαGal per min at 37 °C.

Michaelis–Menten parameters in the hydrolysis of *p*NP glycosides by β-D-xylosidase, β-D-galactosidase and α-D-galactosidase were also determined according to the method of initial velocities as described previously for each enzyme.[6,7,9]

3.3. Enzyme kinetics

Hydrolysis of *p*NPβXyl by the β-D-xylosidase was carried out at 37 °C in 50 mM sodium acetate buffer pH 4.5. The process was initiated by adding a 10 μL aliquot of the enzyme (3.2 mU) to the corresponding substrate solution (0.25–1.0 mM, total reaction volume 200 μL). For the hydrolysis of *p*NPβGal by the β-D-galactosidase, a 50 μL aliquot of the enzyme (1.8–16.8 mU) was added to the substrate solution (0.3–1.3 mM, total reaction volume 320 μL) in 50 mM sodium acetate buffer pH 5.0. The reaction catalyzed by TmGalA was carried out in 50 mM sodium acetate buffer pH 5.0 at 37 °C, the best compromise between maintaining activity of the thermophile-derived enzyme and minimizing spontaneous hydrolysis of *p*NPαGal. Hydrolysis was initiated by adding a 50 μL aliquot of the enzyme (0.2–1.7 mU) to the corresponding substrate solution (105–850 μM, total reaction volume 320 μL).

Kinetic curves for the hydrolysis of the corresponding *p*NP glycoside by each enzyme were recorded with a Hitachi U-3310 spectrometer equipped with a circulating water bath, using 1 cm-path length quartz cuvettes. Specific conditions for each glycosidase-catalyzed reactions are given above. The accumulation of *p*-nitrophenol during the reaction course for 20–50 min was monitored at 365 nm relative to a reference sample containing no enzyme. Each measurement was repeated more than three times. Progress curves for enzyme kinetics were fitted using DYNAFIT software[22] with simple Michaelis–Menten mechanism without inhibition as the input model. K_I^{app} (S_0), k_{cat}^{app}(S_0) plots were done using the program Origin 8.0 (OriginLab Corp., Northampton, MA).

In the DYNAFIT package the symbolism of chemical equations was translated into the underlying systems of ordinary differential equations (ODE) by using the theory of matrices. Each kinetic curve was described by the set of parameters: rate constants, analytic concentrations of reactants, molar response coefficients. Analytic concentrations and instrumental offset of reactants were set variable for the better least-squares minimization. Molar absorption coefficient for *p*-nitrophenol ε=1.48×10^3 $M^{-1}\times cm^{-1}$ (pH 5.0, λ=365 nm) was used as a constant. The rate constants k_1, k_{-1} and k_{cat} defined degree of freedom for the theoretical curve, therefore k_1 was fixed (using value for a diffusion coefficient for small molecules in solution 10^3 mM^{-1} s^{-1}) to provide better result of fitting.

Kinetic constants were calculated by solving the system of ordinary differential equations derived from the Michaelis–Menten kinetics using steady-state assumptions with and without influence of the D-galactose inhibition. The model with mutarotation included additional parameters $k_{\alpha\leftrightarrow\beta}$ and $k_{\beta\leftrightarrow\alpha}$ as the constants, which were obtained in an NMR experiment. The interconversion of the α- and β-galactopyranoses was assumed to be the only important reaction in the mutarotation: the α- and β-furanoses were observed to present to the extent of only 1.7 and 3.2%, respectively.

The competitive inhibition constant for D-galactose was determined from initial rates of hydrolysis of *p*NPαGal obtained over 0.016–0.4 mM concentration and inhibitor concentration over 0.5–3 mM according to the Dixon method.[13]

3.4. Determination of D-galactose mutarotation rate

Mutarotation experiments were carried out using Varian NMR 700 MHz Spectrometer at 37 °C, 25 °C and 4 °C in the buffer solution routinely applied for kinetics (see above) with addition of

10% D_2O. Crystalline α-D-galactopyranose was dissolved directly in an NMR tube to the final concentration 1 mM, placed immediately into the magnet followed by magnetic field homogeneity and solvent signal suppression. To suppress the solvent (H_2O) signals, the pulse sequence with solvent presaturation from standard Varian pulse sequence library was used (relaxation delay $d1$=12.0 s, acquisition time aq=4.9 s, transition number nt=4). ^1H NMR spectra were recorded each minute and concentrations of tautomers were determined by integration of peaks corresponding to anomeric protons (chemical shifts (δ) were 5.26 ppm and 4.58 ppm for the α- and β-pyranose forms; 5.28 ppm and 5.22 ppm for the α- and β-furanose forms of D-galactose, respectively). The curve plot and calculations were made using Origin 8.0 software (OriginLab Corporation, USA). To select relaxation delay d1, longitudinal relaxation times T1 were measured using pulse sequence 'inversion-recovery'. The longest relaxation time T1 was determined for the β-furanose form at 37 °C as 3.3 s and thus, recycling time (aq+d1) was selected as 5T1 and equal 16.9 s.

3.5. Inhibition of TmGalA-catalyzed hydrolysis of pNPαGal by anomers of D-galactose

Experiments were carried out with two types of inhibitor: α-D-galactose and the fully equilibrated D-galactose solution, at a final concentration of 1 mM. To minimize mutarotation, α-D-galactose was dissolved in water kept on ice, and added immediately to the reaction mixture. The equilibrated mutarotation mixture was obtained by incubation of freshly dissolved of α-D-galactose at room temperature for 1 h and then cooled down. The *p*NPαGal hydrolysis reactions were carried out at 4 °C in 20 mM sodium phosphate-citrate buffer pH 5.0, with substrate concentrations between 0.031 mM and 2 mM. The reactions were initiated by addition of 10 μU of pre-cooled TmGalA, and stopped after 20 min by addition of 1 mL of 10% Na_2CO_3; the subsequent protocol has been described before.[9] Inhibition constants were derived from the Lineweaver–Burk plots in the presence and absence of inhibitor.[13]

3.6. Studies of influence of mutarotation on pNPαGal hydrolysis by TmGalA

Rapid *p*NPαGal hydrolysis reactions were monitored by a Hi-Tech Scientific KinetAsyst SF-61 Double Mixing Stopped Flow System (TgK Scientific), equipped with a circulating water bath. Reactions were carried out under routine conditions at fixed substrate concentration at 1 mM. To minimize the effect of mutarotation, hydrolysis was initiated by addition of TmGalA so that complete reaction time did not exceed 5 min. Enzyme amounts in a range of 15–67 mU were used to obtain a set of integral kinetic curves produced in five replicates. The accumulation of *p*-nitrophenol was recorded at 365 nm. Slow *p*NPαGal hydrolysis was monitored by an Eon™ Microplate Spectrophotometer, with an absorbance microplate reader (BioTek Instruments). Progress curves for enzyme kinetics were recorded at 365 nm and *p*-nitrophenol accumulation was monitored as described in the Section 3.3 (**Enzyme kinetics**). Reactions were carried out in a 96-well plate at a fixed substrate concentration of 1 mM. The amount of enzyme was varied within 0.6–10 mU so that the complete hydrolysis time was not less than 15 min, and could be considered as 'long-monitoring-time' reaction. Enzyme progress curves were fitted using DYNAFIT software.[22]

3.7. Docking of D-galactose isomers in the active center of TmGalA

Molecular modeling and docking of α- and β-galactose into the active center of TmGalA were performed using the ICM Pro 3.7 program package.[23,24] See details in the Supplemental data.

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Supplementary data

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