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Self-transferring Product Inhibition Observed during the Hydrolysis of Aryl- β -D-Glucopyranosides by a β -Glucosidase from *Agrobacterium tumefaciens*

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Abstract: The time profile of an enzymatic reaction at an early stage is generally considered to be linear. We observed that the time profile of the hydrolysis of 1 mM *p*-nitrophenyl- β -D-glucoside (pNP-Glc) by a β -glucosidase obtained from *Agrobacterium tumefaciens* (Cbg1) was not linear before 5% of the substrate was consumed, even though the initial concentration of the substrate was much higher than its K_m value. The time profiles obtained with higher concentrations of pNP-Glc suggested that the time profile was the function of the absolute concentration of *p*-nitrophenol (pNP). The addition of various alcohols made the time profile linear. A self-transferring product inhibition model was constructed in which the pNP generated during the hydrolysis acts as an acceptor substrate to inhibit the hydrolysis. The theoretical curve agreed well with the experimental data.

Key words: aryl- β -D-glucoside, β -glucosidase, self-transferring product inhibition, non-linear time profile, transglucosylation

The measurement of the initial rate of an enzymatic reaction is one of the most important experiments used to characterize an enzyme. The initial rate is often estimated by quantifying the increase in the product concentration or decrease in the substrate concentration before 5–10% of the substrate is consumed in the reaction. At this time, the time profile of the reaction is generally considered to be linear. In this report, we describe a non-linear time profile at the early stage of a β -glucosidase-mediated reaction.

The enzyme β -glucosidase from *Agrobacterium tumefaciens* (Cbg1) is a member of the glycoside hydrolase family 3¹⁾ (GH3) enzymes. It was first reported that Cbg1 participated in the *A. tumefaciens* infection of plant cells by hydrolyzing coniferin, a β -D-glucoside of phenylpropanoid.^{2,3)} The substrate specificity of Cbg1 was studied. Cbg1 demonstrated a low K_m value for various aryl- β -D-glucosides but a high K_m for cellobiose, indicating that Cbg1 is a typical aryl- β -D-glucosidase.⁴⁾

Cbg1 possesses a domain architecture similar to that of β -glucosidase from *Cluyveromyces marxianus*,⁵⁾ in which a GH3-catalytic domain [a (α/β)₆-sandwich]⁶⁾ is divided into two parts by the insertion of a PA14 domain consisting of a two-layered β -sheet.⁷⁾ The main role of the PA14 domain in Cbg1 was hypothesized to be the forming of a hydrophobic atmosphere at the acceptor site to make the enzyme suitable

for hydrolyzing hydrophobic glucosides through the characterization of truncated mutants in the region.⁸⁾

We reproducibly observed that the time profile for the hydrolysis of *p*-nitrophenyl- β -D-glucoside (pNP-Glc) by Cbg1 at concentrations much higher than its K_m was not linear, even in the early stage of the reaction. The non-linear profile of the reaction made it difficult to routinely determine its initial rate. We therefore analyzed the non-linear kinetics to determine the reaction mechanism.

MATERIALS AND METHODS

Materials. Four aryl- β -D-glucosides [pNP-Glc, *m*-nitrophenyl- β -D-glucoside (mNP-Glc), *o*-nitrophenyl- β -D-glucoside (oNP-Glc) and phenyl- β -D-glucoside (Ph-Glc)] were purchased from Sigma Chemicals Ltd. (St Louis, USA). All other chemicals were of reagent grade.

Enzymatic reaction. Cbg1 was prepared as previously described.⁸⁾ Enzymatic reactions of Cbg1 were typically carried out in 50 mM 3-morpholinopropanesulfonic acid buffer (pH 6.5) at 30°C, at a concentration of 0.20 μ g/mL in the presence of 0.02% bovine serum albumin as a stabilizer. The enzymatic reaction was stopped by adding an equal volume of 1 M NaCO₃ to quantify nitrophenols or by boiling the reaction for 5 min to quantify glucose. The concentration of each nitrophenol was determined by measuring the absorbance at 405 nm in 500 mM Na₂CO₃, using the calibration curves prepared with each compound. The concentration of D-glucose was quantified by high performance ion exchange chromatography on a CARBOPAC PA1 column (4 $\times$ 250 mm; Dionex Corporation, Sunnyvale, USA) equipped

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Abbreviations: Cbg1, β -glucosidase from *Agrobacterium tumefaciens*; GH3, glycoside hydrolase family 3; pNP-Glc, *p*-nitrophenyl- β -D-glucoside; pNP, *p*-nitrophenol; oNP-Glc, *o*-nitrophenyl- β -D-glucoside; mNP-Glc, *m*-nitrophenyl- β -D-glucoside; Ph-Glc, phenyl- β -D-glucoside.

with a pulsed amperometric detector (DX-3; Dionex Corporation). Chromatography was performed with a linear gradient of 0–0.2 M sodium acetate in 0.1 M NaOH for 20 min, at a flow rate of 1 mL/min.

Data processing. To determine the Michaelis–Menten kinetic parameters for mNP-Glc and Ph-Glc, the initial rates for each substrate were determined at several concentrations ranging from 1/5 to 5 times of each K_m value by quantifying the amount of D-glucose released. The kinetic parameters were calculated by curve-fitting the experimental data to the equation⁹ using Graft ver. 4 (Erithacus Software Ltd., Middlesex, UK). To analyze the time profile of the reaction, a theoretical formula for self-transferring product inhibition was constructed based on the reaction model. Experimental data were regressed on the formula by the curve-fitting method using Graft ver. 4.

RESULTS AND DISCUSSION

Non-linear time profile in the hydrolysis of pNP-Glc.

As shown in Fig. 1, the time course of the release of p-nitrophenol (pNP) from 1 mM pNP-Glc by Cbg1 was not linear within 5% hydrolysis of pNP-Glc, even though the substrate concentration (1 mM) was much higher than its K_m (0.012 mM).⁴ Such a non-linear time profile was not observed at lower substrate concentrations (less than 0.1 mM) within 5% hydrolysis.^{4,8} pNP and D-glucose were the only reaction products observed, which were generated in equimolar amounts.^{4,8} This indicated that the reaction was a simple hydrolysis of pNP-Glc. When the hydrolysis of pNP-Glc at various higher concentrations (up to 10 mM) was examined with the same amount of the enzyme, essentially identical time profiles were obtained (Fig. 2). These results clearly indicated that the ratio of the hydrolysis did not play a role in the non-linearity of the time profile, but the concentration of the product (s) did affect the profile. Therefore, it could be assumed that one of the hydrolytic

products (pNP or Glc) non-competitively inhibited the hydrolysis. Addition of 1 mM D-glucose in the reaction mixture of 1 mM pNP-Glc had no effect on the time course of hydrolysis, indicating that D-glucose was not involved in the inhibition.

Reaction profile in the presence of alcohol.

The time courses for the release of pNP from 1 mM pNP-Glc in the presence of various alkyl alcohols (200 mM concentration) are shown in Fig. 3. The release of pNP was accelerated in the presence of methanol and ethanol, but the time profile of the reaction was not linear. However, in the presence of 1-propanol, 1-butanol, or 1-pentanol, the time profiles were linear with an increase observed in the initial rate. Such effects were also observed in the presence of 2-butanol (200 mM) or 2-methyl-2-butanol (200 mM), which are derivatives of 1-propanol, having secondary and tertiary hydroxyl groups, respectively. The reaction rate with the primary alcohol was the highest, followed by the secondary alcohol, and subsequently, the tertiary alcohol. The difference in the rates was probably because of the difference in transglucosylation rates caused by the reactivity of the classes of alcohols. We already reported that Cbg1 exhibited strong transglucosyl activity from pNP-Glc in the presence of primary alcohols with their chain lengths ≥ 3 .⁴ Cbg1 formed propyl- β -D-glucoside but no detectable D-glucose from pNP-Glc in the presence of 200 mM pNP-Glc.⁸ These results suggest that the high concentration of alcohols with their chain lengths ≥ 3 effectively inhibits the binding of pNP to the acceptor site on Cbg1.

Theory of self-transferring product inhibition.

Considering the above results, a novel pattern of product inhibition, “a self-transferring product inhibition,” was proposed. The scheme of the hypothesis is as follows: pNP formed during the hydrolysis of pNP-Glc acts as the acceptor in the transglucosylation reaction reducing the rate of

Fig. 1. Non-linear time profile observed during the hydrolysis of 1 mM pNP-Glc by Cbg1.

The solid line is the theoretical time course obtained by regressing the data with Equation (6) in the text. The broken line is the tangent line at $t = 0$.

Fig. 2. Time profiles for the hydrolysis of pNP-Glc at various concentrations.

Circles, 1 mM; squares, 2 mM; triangles, 5 mM; diamonds, 10 mM.

Fig. 3. Time profiles of the release of pNP in the presence of various alcohols at 200 mM.

Open circles, none; black squares, methanol; black upward triangles, ethanol; black downward triangles, 1-propanol; black diamonds, 1-butanol; black hexagons, 1-pentanol; gray downward triangles, 2-butanol; open downward triangles, 2-methyl-2-butanol.

hydrolysis. This hypothesis is explained in the two reactions given below which compete with each other.

Reaction (1) represents the hydrolysis of pNP-Glc, and reaction (2) represents the transglucosylation reaction transforming pNP-Glc to pNP [denoted as pNP* to distinguish the origin of pNP in (2)]. It is notable that the substrates and products in reaction (2) are identical, meaning that the reaction is undetectable. The assumption, therefore, does not conflict with the fact that the reaction is a simple hydrolysis. Reaction (2) competes with reaction (1) to decrease the rate of the reaction (1) along with increasing pNP concentration. The product, pNP, is thus considered to act as an inhibitor against the substrate pNP-Glc. Equation (3) represents the rate equation of a normal mixed-type inhibition.

$$d[\text{pNP}]/dt = V_{\max}[\text{pNP-Glc}] / \{K_m(1 + [\text{pNP}]/K_{is}) + [\text{pNP-Glc}](1 + [\text{pNP}]/K_{ii})\} \quad (3)$$

[K_{is} (K_i for slope), the inhibition parameter for competitive term; K_{ii} (K_i for intercept), the inhibition parameter for uncompetitive term]

Considering the experimental conditions where [pNP-Glc] was much higher than its K_m , the competitive term from the denominator $\{K_m(1 + [\text{pNP}]/K_{is})\}$ can be omitted. Thus, Equation (4) is constructed, and is integrated into Equation (5).

$$d[\text{pNP}]/dt = k_{\text{cat}}[E]/(1 + [\text{pNP}]/K_{ii}) \quad (4)$$

$$0.5[\text{pNP}]^2 + K_{ii}[\text{pNP}] - V_{\max}K_{ii}t = C \quad (5)$$

[C: constant of integration]

Considering the boundary condition ($t = 0$, $[\text{pNP}] = 0$), C was calculated as 0. Finally, Equation (6), representing the time profile of the reaction, is obtained by solving Equation (5) for [pNP].

$$[\text{pNP}] = -K_{ii} + (K_{ii}^2 + 2V_{\max}K_{ii}t)^{0.5} \quad (6)$$

It is notable that [pNP] is not a function of [pNP-Glc] in Equation (6), corresponding with the fact that the pattern of

Fig. 4. Time profiles of the hydrolysis of various aryl- β -D-glucosides.

Open circles, pNP-Glc; closed circles, mNP-Glc; open squares, oNP-Glc; closed squares, Ph-Glc.

Table 1. Parameters for various aryl- β -D-glucosides.

	pKa*	K_m (μM)	$V_{\max}$ **	K_{ii} (μM)
pNP-Glc	7.2	13	1.0	87
mNP-Glc	8.4	110	1.8	79
oNP-Glc	7.2	24	2.0	nd***
Ph-Glc	10.0	210	1.0	nd***

*Values of the corresponding aglycons at 25°C. Data are taken from The Merck Index, 12th Ed., Merck Research Laboratory, Whitehouse Station, USA (1996). ** $V_{\max}$ obtained with pNP-Glc was defined as 1.0. ***Not determined due to the linear time profile.

the time course was not affected by the concentration of pNP-Glc (Fig. 2). By regressing the experimental results obtained with 1 mM pNP-Glc with Equation (6), K_{ii} and $V_{\max}$ were calculated to be 0.077 ± 0.012 mM, and 0.0104 ± 0.0007 mM/min, respectively, at the Cbg1 concentration of 0.20 $\mu\text{g/mL}$. The theoretical curve showed a good agreement with the experimental results (Fig. 1), suggesting that the hypothesis well explained the phenomenon. The inhibition parameter (K_{ii}) of pNP was calculated to be very low, suggesting that the high affinity of pNP, one of the hydrolytic products of the pNP-Glc reaction, causes significant deceleration of hydrolysis.

Hydrolysis of aryl- β -D-glucosides.

To investigate whether self-transferring product inhibition is commonly observed with various aryl- β -D-glucosides, time courses for the hydrolyses of oNP-Glc, mNP-Glc and Ph-Glc by Cbg1 were examined. The non-linear time profile was observed with mNP-Glc, but not with oNP-Glc or Ph-Glc (Fig. 4). Kinetic parameters for these compounds are listed in Table 1. The inhibition did not seem to be relate with either the K_m of the substrate or the pKa of the phenolic compounds. Therefore, it was concluded that the self-transferring product inhibitions of Cbg1 are applicable to certain but not all aryl- β -D-glucosides.

Concluding remark.

We analyzed a non-linear time profile obtained in the initial stage of pNP-Glc hydrolysis by Cbg1. The theoretical equation given in this paper may help in the evaluation of enzymatic activities with a similar self-transferring product inhibition pattern.

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