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Note

# Characterization of the subsite structure of the β-glucosidase from *Aspergillus niger*, an aspect of the mechanism of carbohydrate recognition[1]

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**Abstract**

Steady-state kinetics on the reaction catalyzed by the β-glucosidase of *Aspergillus niger* were carried out to evaluate the kinetic parameters, $K_m$ and $k_o$, for phenyl β-D-glucosides. The $k_o/K_m$ values, which may relate to productive binding at subsites, were found to correlate with the substituent constant π (hydrophobicity), suggesting that subsite 2 has a hydrophobic character. A ''hydrophobic-driven'' mechanism is considered to contribute to the productive E–S complex for recognition of the substrate. 

*Keywords:* β-Glucosidase; β-Glucosides; Steady-state kinetics; Subsite structure; Substituent constant π; *Aspergillus niger*

β-D-Glucosidase(EC 3. 2. 1. 21), which catalyzes the hydrolytic reaction mainly for β-(1→4)- and -(1→6)-glucosidic bonds of β-D-glucopyranosides, including cello-oligosaccharides, has received much attention not only for basic studies but also in practical uses [1]. Thoma et al. and Hiromi et al. have hypothesized that the active site of glucohydrolases is composed of subsites, areas that accommodate individual glucose residue of the substrate [2,3]. Based on this subsite theory, the subsite structure namely the number of affinity of subsites, of β-glucosidase from *Aspergillus niger*, has been evaluated by steady-state kinetic studies on a series of *n*-mer oligosaccharide substrates [4].

Withers et al. have studied the kinetics of β-glucosidase-catalyzed reactions for glycosides having 2,4-di- and 4-nitrophenyl aglycons and have described noncovalent E–S intermediates [5,6]. Wijnendaele and De Bruyne have investigated the effect of substituents (phenyl β-xylosides) on β-xylosidase-catalyzed reactions [7]. The effect of the aglycon on the enzyme–glucoside conjugate is considered to be extremely important, and thus we focused on the substituent constant (π) for

Abbreviations: $G_2$, cellobiose; G*n*, cello-oligosaccharides; *n*, degree of polymerization.

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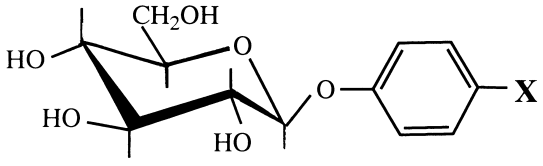
substrate $\beta$-glucosides (**1**) that have a common substituent $x$ at the 4(para)-position of the arylaglycon (Scheme 1). Based on kinetic observations on the enzyme-catalyzed reaction for **1**, we attempted to characterize the subsite structure of the $\beta$-glucosidase of *A. niger*.

## 1. Results and discussion

For evaluation of the kinetic parameters, the Michaelis constant $K_m$ and the molar activity $k_o$ (which is equal to $k_{cat}$), the reaction time-curves were observed for these substrate $\beta$-glucosides (**1**). A typical example of these is shown in Fig. 1, where arbutin (**2**, Scheme 2) was employed as the substrate. The slope of the reaction time-courses, which were obtained at various concentrations of substrate $[S]_o$, gives the initial velocity $v$. Based on a plot of $v$ against $[S]_o$, the kinetic parameters $K_m$ and $k_o$ can be determined by the Michaelis–Menten equation.

The $K_m$ and $k_o$ values for the $\beta$-glucosidase catalyzed reactions were obtained for various glucosides (**1**), as summarized in Table 1, and show that these values are characteristic for their substituents $x$. The value of $k_o/K_m$ was evaluated for each **1** and




Scheme 1.

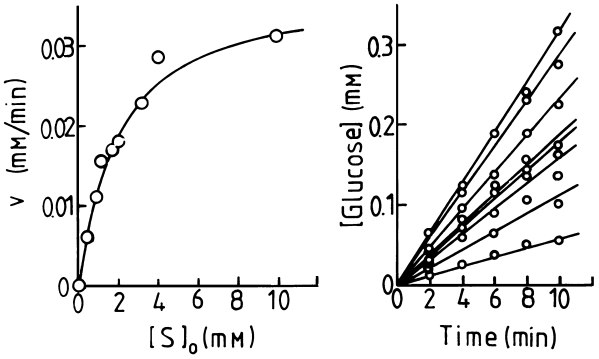
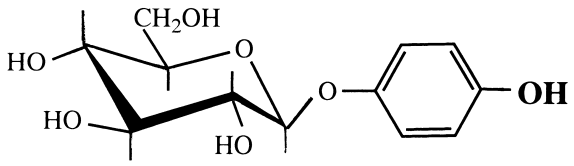



Fig. 1. The $\beta$-glucosidase-catalyzed reaction for the substrate **2**. Right: reaction time curves; left: a plot of the initial velocity $v$ against the concentration of substrate (**2**) $[S]_o$. [E]; $2.53\times10^{-8}$ M, pH 5.0, 25.0 °C. The solid (data-fitted) lines were obtained theoretically by using personal computer software.




**Arbutin**

Scheme 2.

the results are presented in Table 1. These suggest that $k_0/K_m$ is apparently dependent on the substituent $x$, and it is possible that there is some relation between $k_0/K_m$ and the characteristic $x$. Here we note the hydrophobic character of $x$, because Fujita et al. have proposed a new substituent constant $\pi$, which is related to the hydrophobicity of substituents and is derived on the basis of partition coefficients [8]. Thus, we examined a plot of $k_o/K_m$ against $\pi$ of $x$ for these glucosides (**1**). As illustrated in Fig. 2 (open circles), an excellent correlation was found between $k_o/K_m$ and $\pi$. In this study, aglycons having substituent $x$ at the 4(para)-position were employed exclusively in the reaction, to avoid more complicated interpretations (effects other than hydrophobicity, such as electronic, steric, charge, and so on). Another kinetic observation was carried out with *o*-nitrophenyl $\beta$-glucoside for a reference. Its $k_o/K_m$ value was plotted against $\pi$, as shown in Fig. 2 (closed circle), and indicated a good consistency with results for **1**. As many aryl $\beta$-glucosides having appropriate substituents at the 4-position are not readily available, we examined only four glucosides. Although kinetic studies with $\beta$-glucosidases have been reported by many researchers, the rate parameters $K_m$ and $k_o$ have been seldom obtained for $\beta$-glucosides [7,9,10]. Based on the reported values for the $\beta$-glycosidase-catalyzed reactions for $\beta$-glucoside substrates [5,11,12], $k_o/K_m$ values have been evaluated to examine the relation with $\pi$, as illustrated in Fig. 3 (points). These reported values are mostly consistent with our experimental results.

The subsite structure of $\beta$-glucosidase (*Asp. niger*) suggests that there are two main binding modes of **1**, one productive ($p$) and one non-productive ($q$) as schematically illustrated in Fig. 3, where subsite affinities at subsites 1 and 2, $A_1$ and $A_2$, are much larger than those of the others $A_i (i=3\sim6)$, as described elsewhere [4]. Based on the subsite theory proposed by Hiromi et al. [3,13],

Table 1
Kinetic parameters on the β-glucosidase-catalyzed hydrolysis for β-glucosides **1**

| (Glucoside) | $\pi$ | $k_o$($s^{-1}$) | $K_m$ (mM) | $k_o/K_m$ ($s^{-1}\,mM^{-1}$) |
|---|---|---|---|---|
| **1** (x = $NO_2$) | 0.50 | 270 ± 30 | 0.57 ± 0.04 | 480 |
| **2** (x = OH) | −0.87 | 20 ± 4 | 2.1 ± 0.4 | 10 |
| **1** (x = H) | 0 | 340 ± 10 | 3.5 ± 0.2 | 100 |
| *o*-Nitrophenyl β-glucoside | 0.33 | 360 ± 20 | 1.4 ± 0.1 | 250 |
| Cellobiose* | — | 360 | 0.85 | 440 |

[E], $2.5\times10^{-8}$ M, 0.02 M acetate buffer, pH 5.0, 25.0 °C. Each aglycon of the glucosides **1** have the appropriate substituents *x* at C-4 of the aryl group.
* The kinetic parameters have been described in detail elsewhere [4]; $\pi$, reported value [8].

the $k_o/K_m$ value for an *n*-mer substrate is given by the sum of the $A_i$ affinities of the *i* subsites occupied by the binding of the substrate molecule, $\sum_i^{cov.}$

$$(k_o/K_m)_n = (0.018)k_{int}\sum_p \exp\left(\sum_i^{cov.} A_i/RT\right)_{n,p.} \quad (1)$$

where 0.018 arises from the contribution of the mixing entropy in water at 25 °C (2.4 kcal $mol^{-1}$) and $k_{int}$ is assumed to be constant irrespective of *n* and *j* (binding modes, $j=p+q$), and R and T are the gas constant and temperature, respectively. Thus, only the productive (*p*) term out of the total binding modes (*j*) is involved in the value of $k_o/K_m$ as represented in eq (1). Focusing on $k_o/K_m$ we considered that the productive binding (*p*) required that the aglycon of **1** be bound at subsite 2. As seen in Fig. 2, $k_o/K_m$ was found to correlate with $\pi$, this strongly suggesting that the hydrophobicity ($\pi$) of the aglycon takes a substantial role in the binding at subsite 2, as schematically illustrated in Fig. 3. Subsite 2 is suggested to be hydrophobic.

The value of $k_o/K_m$(= 440 $mM^{-1}\,s^{-1}$) for the best substrate, cellobiose $G_2$, is nearly equal to that (= 480 $mM^{-1}\,s^{-1}$) for **1**(X = $NO_2$), as shown in Table 1. This finding means that the "aglycon" (glucose residue) of $G_2$ has a $\pi$ value almost identical to that of **1** (x = $NO_2$). Hiromi et al. have proposed that hydrogen bonds, formed between OH groups of the glucose residue and amino acid residues of the subsite contribute to the E–S complex [14]. However, it is quite possible that hydrophobicity drives the β-glucoside into the E–S conjugate. If so, hydrophobicity may contribute decisively to carbohydrate recognition through conjugate (productive complex) formation between subsite and glucose residue in substrate cello-oligosaccharides. Hydrogen bonds may play an important role in the fixation of a glucose residue to a subsite. Hydrophobicity may contribute by drawing a glucose residue toward a subsite, an effect here called "hydrophobic-driven".

Intensive investigations on the β-glucosidase-catalyzed reaction with the β-glucoside substrates have been carried out to clarify its substrate specificity [1,15–17], but they do not describe such a hydrophobic-driven mechanism for formation of the E–S complex. At this stage, we do not have

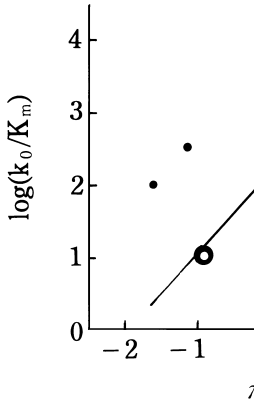



Fig. 2. The plot of log ($k_o/K_m$) was obtained from the kinetic parameters, $K_m$ and $k_o$ as shown in Table 1. The substituent constant $\pi$ is the value reported by Fujita et al. [8]. ●, *o*-nitrophenyl β-glucoside; •, evaluated using the reported values in the literature [5,11,12].

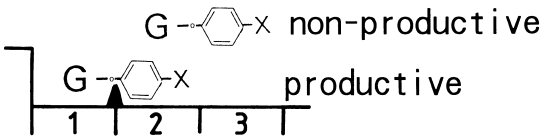



Fig. 3. Binding modes of glucosides on the subsites of β-glucosidase. There are two main modes, one productive and one non-productive binding. G, glucose residue; *x*, substituent; closed triangle; the catalytic site.

other reliable evidence or a final conclusion on this possibility. Experimental work on the effect of organic solvents [18], and on transient-phase kinetics by the stopped-flow method for the hydrophobic-driven E–S conjugate formation, is currently in progress.

## 2. Experimental

*Materials*.—The enzyme preparation β-glucosidase from *A. niger* was purchased from Novo Nordisk Bioindustry Co. Ltd., Tokyo, and was highly purified by column chromatographic procedures to homogeneity as described previously [19]. The concentration of the enzyme was determined spectrophotometrically, taking $A^{1\%}_{280\,nm}$ as 15.4 $cm^{-1}$ and Mw as 137,000. β-Glucosides **1**; *p*-nitrophenyl β-glucoside (**1**, x = $NO_2$), arbutin (**2**), phenyl β-glucoside (**1**, x = H), and *o*-nitrophenyl β-glucoside were purchased from Sigma Co. and were used without further purification. Cellobiose $G_2$ and cello-oligosaccharides $G_n$ were prepared from a hydrolyzate of Avicel as described elsewhere [4] and were confirmed to be 98% pure by HPLC. An enzymatic glucose determination-kit, Glucose C-II Test W, was purchased from Wako Pure Chemicals Co. and used with twice dilution with 0.02 M phosphate buffer, pH 7.1. Other chemicals, guaranteed grade, were obtained from Nacalaitesque Inc. and were used without further purification.

*Steady-state assay of the* β-*glucosidase-catalyzed hydrolysis for glucosides*.—The β-glucosidase-catalyzed hydrolytic reactions for $G_2$ and β-glucosides (**1**), were carried out in 0.02 M acetate buffer, at pH 5.0 and 25 °C: Solutions of enzyme (60 μL), and buffer (120 μL) were mixed immediately, and aliquots (40 μL) were taken out at appropriate time-intervals and mixed with 0.5 M NaOH solution to stop the reaction. The quantities of glucose liberated from the substrate glucosides were determined with the enzymatic glucose determination reagent at $A_{505\,nm}$, using the calibration curve for glucose. A plot of the glucose released against time gives the reaction time-curve.

*Determination of the kinetic parameters*.—Based on the reaction time-curve observed, whose slope gives the initial velocity $v$, the kinetic (rate) parameters, namely the Michaelis constant $K_m$ and the molar activity $k_o$ can be determined for the substrate β-glucosides **1**. With **1** (x = $NO_2$) as a substrate, $v$ was confirmed to be a purely hydrolytic reaction and does not involve any transfer reaction under the experimental conditions employed [18]. The kinetic parameters and the standard deviations, represented by ±, were obtained by using a NEC Personal Computer PC-9801 and Bio-Graph Software (Kyoto Soft Co.).

## 3. Supplement

Subsite structure may be expressed by -$n$/+$n$ (or a/b), as presented in other papers, in fact, these indications have been used since 1953 and are sometimes convenient [20,21]. We do not deny the practical use of this expression in the field of proteases and amylases. However, this nomenclature contains an essential defect. As already described on a theoretical basis [3,13], the number of subsites must be expressed systematically and with numerical unification. For example, eq (1) is quite difficult to explain with the $-n/+n$ (or a/b) notations, but it is reasonably explained with the numerical values ($i$ = 1,2,3,...). Thus, the nomenclature $i$ is indispensable for a theoretical description of the subsite structure. Based on the consideration, we have used the nomenclature $i$ for the subsite structure in this study.

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