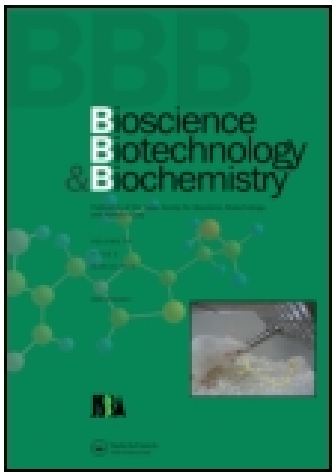


Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 12 Jun 2014.

To cite this article: Rudee Surarit, Hirokazu Matsui, Seiya Chiba, Jisnuson Svasti & Chantragan Srisomsap (1997) Evidence for a Single Active Site in β-d-Glucosidase/β-d-Fucosidase from Dalbergia cochinchinensis Seeds, Bioscience, Biotechnology, and Biochemistry, 61:1, 93-95, DOI: 10.1271/bbb.61.93

To link to this article: http://dx.doi.org/10.1271/bbb.61.93

*Biosci. Biotech. Biochem.*, **61** (1), 93-95, 1997

# Evidence for a Single Active Site in β-D-Glucosidase/β-D-Fucosidase from *Dalbergia cochinchinensis* Seeds

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Received June 24, 1996

**Kinetic analyses have been done on the hydrolysis of *p*-nitrophenyl β-D-glucoside (PNPG) and *p*-nitrophenyl β-D-fucoside (PNPF) by the β-D-glucosidase/β-D-fucosidase enzyme from Thai Rosewood (*Dalbergia cochinchinensis* Pierre). PNPF showed a competitive inhibition of PNPG hydrolysis with a $K_i$ of 0.42 mM. Hydrolysis of mixtures of PNPG and PNPF at fractional ratios ranging from 0 to 1 showed Lineweaver-Burk plots intermediate between the two extremes. The apparent $K_m$ and apparent $V_{max}$ at each fractional ratio showed good correspondence with the theoretical curves predicted for the existence of a single common active site for the hydrolysis of the two substrates.**

**Key words:** active site; β-D-glucosidase; β-D-fucosidase; *Dalbergia cochinchinensis*



An enzyme containing both β-D-glucosidase (EC 3.2.1.21) and β-D-fucosidase (EC 3.2.1.38) activities, present in large amounts in the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre), has been purified to electrophoretic homogeneity.[1,2] Interestingly, this enzyme has a higher substrate-binding affinity but lower rate of hydrolysis for β-D-fucoside than for β-D-glucoside.[2] The $K_m$ and $V_{max}$ for *p*-nitrophenyl β-D-glucoside (PNPG) were 4.7 mM and 50 μmol/mg protein/min, and those for *p*-nitrophenyl β-D-fucoside (PNPF), 0.57 mM and 21 μmol/mg protein/min. Based on the 3.7-fold higher $V_{max}/K_m$ ratio for PNPF compared to PNPG, it has been suggested that the enzyme should be designated as a β-D-fucosidase rather than a β-D-glucosidase.[2] In addition, under suitable conditions of high monosaccharide concentration and elevated temperature, the hydrolytic reaction of this enzyme can be reversed, leading to a net synthesis of disaccharides and trisaccharides.[3] Studies on the active site of the enzyme may be useful for understanding the catalytic mechanism of the enzyme and for improving the synthesis of oligosaccharides.

Some glucosidases have been shown to hydrolyze other glycosides, such as fucosides, and in some cases, the reaction with different substrates occurs by means of multiple active sites. Thus, rat liver lysosomal acid α-D-glucosidase has been reported to contain at least two active sites,[4] while almond emulsin β-D-glucosidase has been reported to have one catalytic site and two binding sites.[5] On the other hand, Chiba *et al.* have reported that several glucosidases with specificity for a wide range of substrates have a single active site for different substrates. For instance, each α-glucosidase from buckwheat,[6] sugar beet,[7] pig liver,[8] rabbit muscle,[9] and rabbit liver[10] has a single active site catalyzing the hydrolysis of low and high molecular weight substrates such as maltose and α-glucan (soluble starch or glycogen).

It has also been recently reported that cassava linamarase has a single active site for the hydrolysis of β-D-glucoside and β-D-fucoside.[11] Unfortunately, there have been few reports on β-D-fucosidases, and the active sites of these enzymes have not been identified. In our previous paper,[12] we reported the evidence from chemical modification studies of the enzyme using conduritol B epoxide, which suggest that the Thai Rosewood enzyme has a single active site for both β-D-glucoside and β-D-fucoside.

For this paper, kinetic analyses were done to confirm the results of our previous studies and to demonstrate clearly that the enzyme has a single active site responsible for the hydrolysis of PNPG and PNPF.

## Materials and Methods

*Chemicals.* PNPG and PNPF were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). "Glucose AR II" kit was a product of Wako Pure Chemical Co., Japan. Other chemicals were of laboratory grade.

*Enzyme.* The enzyme was purified by the procedure described in the previous paper,[1,2] and was homogeneous on both non-denaturing and SDS polyacrylamide gel electrophoresis.

*Enzyme assay.* The standard assay reaction mixture, consisting of 0.2 ml of each substrate at various concentrations, 0.2 ml of 0.1 M sodium acetate buffer, pH 5.0, and 0.1 ml of purified enzyme solution, was incubated at 37°C for 10 min. After incubation of this reaction mixture, 2 ml of 1.3 M $Na_2CO_3$ was added and the *p*-nitrophenol (PNP) liberated was measured spectrophotometrically at 400 nm. The glucose liberated was measured by the Tris-glucose oxidase-peroxidase method[13] using a "Glucose AR II" kit.

*Mixed substrate reaction.* The enzyme assay was done under the standard conditions. The initial velocity $v$ was measured by the PNP or glucose liberated at each of the total substrate concentrations $s$, which represent the sum of the concentrations of PNPG and PNPF. The fraction $f$ of PNPG in the substrate is indicated by the fractional weight: $f = [PNPG]/([PNPG]+[PNPF])$.

## Results

### *Course of hydrolytic activities*

The course of the reaction was studied under the standard conditions as shown in Fig. 1. When the β-D-glucosidase activity of the enzyme was measured, the amount of PNP

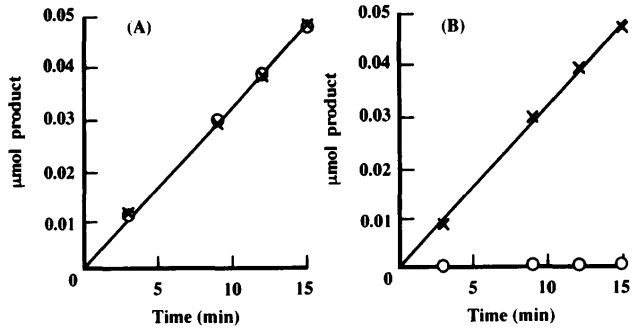


**Fig. 1.** Course of the Hydrolytic Activities of β-D-Glucosidase/β-D-Fucosidase towards PNPG and PNPF.

Standard assay conditions were used with either 5 mM PNPG (A, 0.13 μg of enzyme) and 1 mM PNPF (B, 0.26 μg of enzyme) as substrates. The liberation of *p*-nitrophenol (- × -) and glucose (— ○ —) were measured spectrophotometrically as described in Materials and Methods.

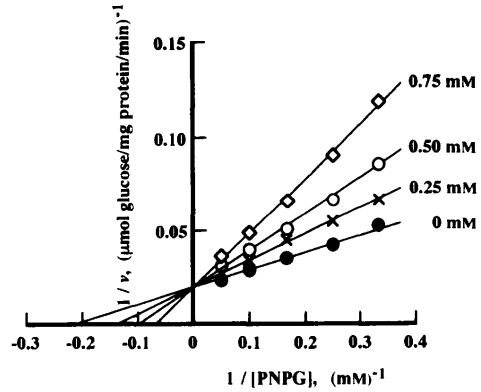


**Fig. 2.** Inhibition of β-D-Glucosidase Activity by PNPF.

The reaction mixture, containing 0.25 ml of substrate (PNPG) at various concentrations, 0.1 ml of 0.1 M sodium acetate buffer, pH 5.0, 0.1 ml of various concentrations of PNPF, and 0.05 ml of enzyme solution, was incubated at 37 C for 10 min. The glucose liberated was measured using a "Glucose AR II" assay kit.

and glucose liberated from PNPG were equal over 15 min. When the β-D-fucosidase activity was measured by PNP released from PNPF, this reaction was also linear over 15 min, with the fucose liberated having no effect on the measurement of glucose using the "Glucose AR II" kit.

*Competition between substrates*

The β-D-glucosidase activity of the enzyme was inhibited by PNPF. When glucose liberation was followed using various concentrations of PNPG in the presence of different concentrations of PNPF, the Lineweaver–Burk plots clearly showed competitive inhibition by PNPF of the β-D-glucosidase activity, with a $K_i$ of 0.42 mM (Fig. 2). This was almost equal to the $K_m$ for PNPF, 0.57 mM. This enzyme also catalyzes the transglycosylation reaction at higher substrate concentrations, but such phenomena were not seen in these conditions.

*Kinetic behavior with mixed substrates, PNPG and PNPF*

When an enzyme acts on a mixture of two substrates to produce a common product, the kinetic features would differ depending on whether the two substrates were hydrolyzed at a single active site, or at two active sites, each specific for one substrate.[6-10,14] This question may be analyzed by studying the kinetic properties as described in detail in other studies.[14] The main features that provide conclusive

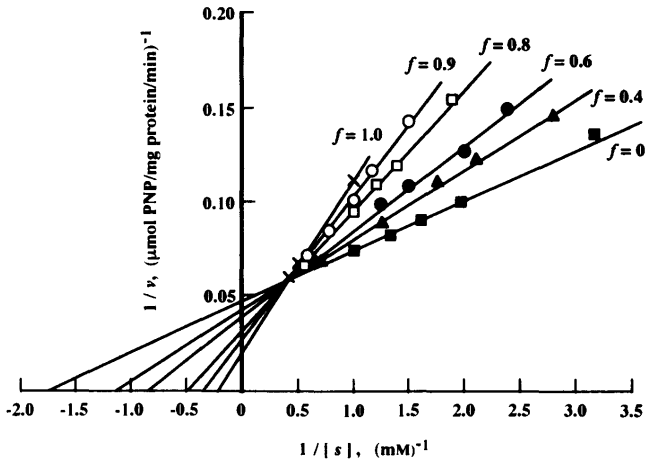


**Fig. 3.** Plots of $1/v$ *versus* $1/s$ for Mixtures of PNPG and PNPF Substrates.

Each reaction mixture, containing 0.05 ml of enzyme, 0.05 ml of 0.4 M sodium acetate buffer, pH 5.0, and 0.4 ml of the mixed substrates was incubated under standard conditions. The mole fraction of each substrate in the mixture is given by: $f$= [PNPG]/([PNPG]+[PNPF]).

information about the active site are: 1) the possibility of competition between the two substrates, 2) the linearity of double reciprocal plots, and 3) the dependence of $V_{max}$ on the fraction of the two substrates.

Lineweaver–Burk plots for the hydrolysis of the mixed substrates, PNPG and PNPF at different mole fractions ($f$), are shown in Fig. 3, where $v$ is the initial velocity for the total substrate concentration, and $s$ is the sum of the concentrations of the two substrates. The fraction of each substrate is indicated by weight: $f$=[PNPG]/[PNPF]+[PNPG]), so that at $f$=1, only PNPG is present and at $f$=0, only PNPF is present. The results clearly show that the graphs between $1/v$ against $1/s$ were linear at each $f$ fraction and indicate that there was a competition between the two substrates. The $V_{max}$ calculated from each straight line also changed continuously with $f$, without showing any maximum or minimum. Such features are characteristic of enzymes which have a single active site for reaction with two substrates.

If the reactions of substrate A and substrate B are catalyzed at the same active site of an enzyme E, the reaction may be written as follows:

$$\mathrm{E+A+B} \underset{k_{-A}}{\overset{k_{+A}}{\rightleftharpoons}} \mathrm{EA} \xrightarrow{k_{+1}} \mathrm{E+P}$$
$$\mathrm{E+A+B} \underset{k_{-B}}{\overset{k_{+B}}{\rightleftharpoons}} \mathrm{EB} \xrightarrow{k_{+2}} \mathrm{E+P}$$

At the steady state, the initial rate $v$ for the formation of the product P can be expressed by the following formula:

$$v=\frac{V_1K_A[\mathrm{A}]+V_2K_B[\mathrm{B}]}{1+K_A[\mathrm{A}]+K_B[\mathrm{B}]}=\frac{\dfrac{V_1K_Af+V_2K_B(1-f)}{K_Af+K_B(1-f)}+s}{\dfrac{1}{K_Af+K_B(1-f)}+s}$$

where

$$K_A=\frac{k_{+A}}{k_{-A}+k_{+1}}\ ;\quad K_B=\frac{k_{+B}}{k_{-B}+k_{+2}}$$

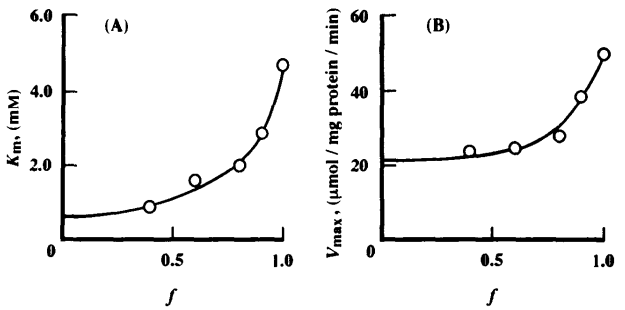


**Fig. 4.** Dependence of $K_m$ (A) and $V_{max}$ (B) on $f$ for the Mixed Substrate Reactions.

. theoretical values; —○ –, experimental values.

$$V_1 = k_{+1}[E_0]; \quad V_2 = k_{+2}[E_0]$$

$$s = [A] + [B] \quad \text{and} \quad f = \frac{[A]}{[A] + [B]}$$

If PNPG and PNPF occupy a common active site, the apparent Michalelis constant $K_m$ and the apparent maximum velocity $V_{max}$ may be expressed as functions of $f$ by the following formulae[14]:

$$K_m = \frac{1}{K_A f + K_B(1-f)}$$

$$V_{max} = \frac{V_1 K_A f + V_2 K_B(1-f)}{K_A f + K_B(1-f)}$$

where $K_A$ and $K_B$ are the reciprocals of the $K_m$ for PNPG and PNPF, while $V_1$ and $V_2$ are $V_{max}$ at $f=1$ and $f=0$, respectively. Using the equations, the results obtained in Fig. 4 indicate excellent agreement between the experimental values and the theoretical values for both PNPG and PNPF. These data strongly suggest that the enzyme contained a single active site for hydrolysis of both the substrates tested.

## Discussion

We have previously reported studies on the chemical modification of the β-D-glucosidase/β-D-fucosidase from *Dalbergia cochinchinensis* Pierre by conduritol B epoxide, which suggest that the enzyme has a single active site for the two substrates, PNPG and PNPF.[12] This study provides strong evidence using a kinetic approach for the existence of a single active site for the hydrolysis of PNPG and PNPF. PNPF also exhibits competitive inhibition towards the β-D-glucosidase activity of the enzyme, with a $K_i$ of 0.42 mM, similar to the $K_m$ (0.57 mM) for PNPF. This also implies that PNPG and PNPF would bind competitively to the same site on the enzyme molecule.

The kinetic approach has been widely used to find whether two substrates are hydrolyzed at a single active site or at two active sites, one specific for each substrate. Hiromi *et al.*[14] have discussed the kinetic method in some detail in their study of *Rhizopus delemar* glucoamylase. In their analysis,[14] if the enzyme catalyzed two substrates using a single active center, the substrates could compete with each other, double reciprocal plots of $1/v$ and $1/s$ would be linear, and the variation of $V_{max}$ with $f$ would be a series of straight lines that showed continuous changes from $V_1$ to $V_2$, with no maximum, for values of $f$ ranging from 0 to 1. On the other hand, if two active sites were involved in the mechanism of action of enzyme, the linearity between $1/v$ and $1/s$ would not be observed, and $V_{max}$ could have other relationships of $f$. By applying this theory to study the active site of β-D-glucosidase/β-D-fucosidase from the seeds of *Dalbergia cochinchinensis* Pierre, we have found that the competition between two substrates, the linearity of the plots between $1/v$ and $1/s$, and the dependence of $V_{max}$ on $f$, were consistent with the results theoretically predicted for a single active site catalyzing the hydrolysis of the two substrates, PNPG and PNPF.

Cassava linamarase, another β-D-glucosidase with hydrolytic activity towards both glucosides and fucosides, has recently been reported to contain a single active site.[11] Although our enzyme shows some similarities in activity to cassava linamarase, the enzyme from *Dalbergia cochinchinensis* Pierre could not hydrolyze linamarin, and the production of cyanide from cyanogenic glycosides has not been detected.[2] From the studies, it is still not known how many subsites are involved in the binding of substrates. However, the enzyme has been found to be of potential use for the synthesis of oligosaccharides using the reverse hydrolytic reaction[1,3] so that knowledge about the nature of active site of the enzyme may be useful for improving the synthetic reaction and the production of novel oligosaccharides. Further studies will also be required to identify the active site of the enzyme and the natural substrate(s), so that the physiological importance of the enzyme may be known.

*Acknowledgments.* This study was supported by the Chulabhorn Research Institute and by the Japan Society for Promotion of Science-National Research Council of Thailand (JSPS-NRCT) Collaborative Program.

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