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Isolation and Characterization of an Enzyme with β-Glucosidase and β-Fucosidase Activities from *Dalbergia cochinchinensis* Pierre[1]

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A glycosidase enzyme with both β-glucosidase and β-fucosidase activities has been purified from the seeds of *Dalbergia cochinchinensis* Pierre (Thai Rosewood) by ammonium sulfate fractionation, preparative isoelectric focusing, and Sephadex G-150 chromatography. The enzyme has molecular weights of 330,000 in the native state and 66,000 in the denatured state. Hydrolysis of *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside showed pH optimum at pH 5.0 and was inhibited by δ-gluconolactone, $HgCl_2$, and *p*-chloromercuribenzoate. The K_m and k_{cat} values of the purified enzyme were 5.4 mM and 307 s^{-1} for *p*-NP-β-D-glucoside and 0.54 mM and 151 s^{-1} for *p*-NP-β-D-fucoside, so that the latter had by far the higher k_{cat}/K_m ratio. *p*-NP-β-D-galactoside, *p*-NP-β-D-xyloside, and *p*-NP-α-L-arabinoside were hydrolyzed more slowly. Hydrolysis of sophorose, laminaribiose, and gentiobiose were also rather slow, and hydrolysis of cellobiose was even slower. No hydrolysis of the cyanogenic glucosides linamarin or prunasin, but some hydrolysis of amygdalin and salicin was found. Further studies are required to identify the natural substrates of the enzyme. However, high yields, ease of purification, and storage stability of the enzyme make it a useful candidate for various applications, such as study of oligosaccharide synthesis by reversal of hydrolysis.

Key words: *Dalbergia* spp., β-fucosidase, β-glucosidase, glycosidase, rosewood.

Glycosidases catalyze the hydrolysis of glycosidic linkages formed between the hemiacetal hydroxyl group of a cyclic aldose or ketose and the hydroxyl group of another compound (*1*). These enzymes have potential applications for sequence determination of oligosaccharides (*2-4*), as well as for oligosaccharide synthesis by reversal of their hydrolytic action (*5-8*). For these reasons, seeds from more than 50 species of Thai plants have been screened for nine glycosidase enzymes using *p*-NP-glycosides as substrates (*9*). The results showed very high levels of β-glucosidase and β-fucosidase activities in the seeds of *Dalbergia cochinchinensis* Pierre (Thai Rosewood) compared to the levels of these or other glycosidase enzymes found in the seeds of other plant species.

The β-glucosidases [EC 3.2.1.21] are a heterogeneous group of enzymes with various functions (*10*), including the hydrolysis of cyanogenic glycosides (*11-14*), cellobiose (*15*), gentiobiose (*16*), or phenolic glucosides (*17*). Moreover, some β-glucosidases of well-defined specificity have been classified in separate categories, such as amygdalin β-glucosidase [EC 3.2.1.117] (*18*), prunasin β-glucosidase [EC 3.2.1.118] (*19*), and thioglucosidase [EC 3.2.3.1] (*20*). β-Fucosidases [EC 3.2.1.38] are less common and their functions are less well known (*21, 22*). Moreover, some β-glucosidases can hydrolyze β-fucosides (*13*), and some β-fucosidases can hydrolyze β-glucosides (*21*). This paper reports the purification of an enzyme with both β-glucosidase and β-fucosidase activities from the seeds of *D. cochinchinensis* Pierre, and characterization of the substrate specificities and other properties of the enzyme.

MATERIALS AND METHODS

Materials—Seeds of *D. cochinchinensis* Pierre (Thai Rosewood) were kindly provided by the ASEAN-Canada Forest Tree Seed Center, Muaklek, Saraburi, Thailand. *p*-NP-glycosides, 4-MU-glycosides, gentiobiose, cellobiose, glucono-1,5-lactone, linamarin, amygdalin, prunasin, laminarin, phloridzin, arbutin, sinigrin, *p*-chloromercuribenzoate, molecular weight standards for SDS-polyacrylamide gels and for gel filtration, polyvinyl-polypyrrolidone, and phenylmethylsulfonyl fluoride were from Sigma Chemical, St. Louis, MO, USA. Sophorose was from Dextra Laboratories (Reading, UK), and laminaribiose was from Sei-

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Abbreviations: *p*-NP-, *para*-nitrophenyl-; 4-MU-, 4-methyl-umberriferyl-.

kagaku (Japan).

Enzyme Purification—Seeds were surface-sterilized with sodium hypochlorite, steeped overnight in distilled water, and homogenized in 2 volumes of 0.05 M sodium acetate buffer, pH 5.0, containing 1 mM phenylmethylsulfonylfluoride and 5% (w/v) polyvinyl-polypyrrolidone. The homogenate was centrifuged at 12,000 × *g* and 25% (w/v) Dowex 2-X8 was added to the supernatant, followed by gentle stirring and removal by suction. The crude extract obtained was fractionated by ammonium sulfate at 35-75% saturation, and the precipitate was resuspended in 0.1 M sodium acetate buffer, pH 5.0. The resuspended precipitate was dialyzed against distilled water overnight and fractionated by preparative isoelectric focusing in a Rotofor apparatus (Bio-Rad, Hercules, CA, USA) using 2% ampholyte (with ratio pH 4-6: pH 3-10 of 5 : 1) for 4 h at 12 W. Fractions containing β-glucosidase and β-fucosidase activity were pooled, concentrated by ultrafiltration, and subjected to chromatography in a Sephadex G-150 column (2.5 cm × 80 cm), equilibrated with 0.1 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl at a flow rate of 21 ml/h. Fractions containing glycosidase activity were pooled, dialyzed against distilled water and lyophilized.

Measurement of Enzyme Activity and Protein Content—Hydrolysis of *p*-NP-glycosides was performed in 0.1 M sodium acetate buffer, pH 5.0, at 30°C, and released *p*-nitrophenol was measured at 400 nm (*23*). Kinetic studies were analyzed by using the Enzfitter computer program (Elsevier Biosoft, Cambridge, UK) using non-linear regression or linear regression. Hydrolysis of various low molecular weight natural substrates was followed by measuring glucose release with a commercial glucose oxidase kit (BM Laboratories, Bangkok, Thailand). With cyanogenic glycosides, release of HCN was also determined by trapping with picric acid (*13*). Hydrolysis of the [β(1,3)] glucose polymer laminarin was assayed using *p*-hydroxybenzoic hydrazide (*24*). Protein content was generally determined by the Coomassie Blue dye binding method (Bio-Rad), but column effluents were screened by measurement of A_{280}.

Analytical Gel Electrophoresis and Analytical Gel Filtration—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (*25*), using a 3% acrylamide stacking gel and a 7.5% acrylamide separating gel (7.5 × 5.0 cm). The molecular weight of the purified enzyme in the denatured state on this system was estimated using rabbit muscle myosin (205 k), *Escherichia coli* β-galactosidase (116 k), rabbit muscle phosphorylase *b* (97.4 k), bovine serum albumin (66 k), ovalbumin (45 k), and carbonic anhydrase (29 k) as standards. Non-denaturing electrophoresis was performed in 5% polyacrylamide slab gels (*6*), and gels were stained for protein with Coomassie Blue R-250 and for activity with 4-MU-glycosides in 0.1 M sodium acetate buffer, pH 5.0. The native molecular weight of the enzyme was determined by gel filtration on a Sephacryl S-200 column (1.8 × 90 cm) in 0.2 M NaCl, 10 mM potassium phosphate buffer, pH 7.0, using β-amylase (200 k), bovine serum albumin (66 k), ovalbumin (45 k), and cytochrome *c* (12.4 k) as standards.

HPLC Analysis of Hydrolysis Products—On occasions, reaction products after enzymatic hydrolysis were characterized by HPLC (Waters 625 LC) using an Aminex HPX-87C column at 85°C, eluted with water and connected to a refractive index detector (Waters 410).

RESULTS AND DISCUSSION

Purification of β-Glucosidase/β-Fucosidase from Thai Rosewood—Crude extracts of *D. cochinchinensis* Pierre (Thai Rosewood) contained high levels of hydrolytic activity towards both *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside. Both activities were therefore followed during the course of purification. When the resuspended and dialyzed 35-75% ammonium sulfate precipitate was fractionated by preparative isoelectric focusing on a Rotofor apparatus (Fig. 1), both enzymatic activities were eluted in the same peak, with pI about 5.6. The A_{280} profile is not shown because it was rather broad, containing not only protein, but also pigments. Tubes 8-14 containing both enzyme activities were pooled, concentrated by ultrafiltration to a volume of about 5 ml, then fractionated by gel filtration on a Sephadex G-150 column. Both β-glucosidase and β-fucosidase activities were eluted in the same position (Fig. 2), close to the void volume of the column and well separated from the majority of other proteins. The gel filtration pool (Tubes 26-38) was dialyzed against distilled water and lyophilized. A summary of the purification procedure is shown in Table I, indicating that the purification and yield for both β-glucosidase and β-fucosidase activities were very similar at each step, with the overall yield of both enzymes being about 30%. The high levels of both enzyme activities in the seed (250-350 nkats/g seed) enabled the purification to be achieved with a simple series of steps, with a yield of about 1 mg of enzyme per 10 g of seed.

Analytical Gel Electrophoresis and Analytical Gel Filtration—The purified enzyme was shown to exhibit a single band of molecular weight 66,000 on SDS-polyacrylamide gel electrophoresis (Fig. 3A). However, since gel filtration of the native enzyme on Sephacryl S-200 gave a molecular weight of 330,000, the native enzyme was believed to consist of 4-6 subunits. The subunit molecular weight is similar to that of many cyanogenic β-glucosidases (linamarases), which range from 59,000 to 65,000 (*11-14*), but in the native state, the latter enzymes vary considerably in size, consisting of 2 subunits in butter bean (*12*), 10

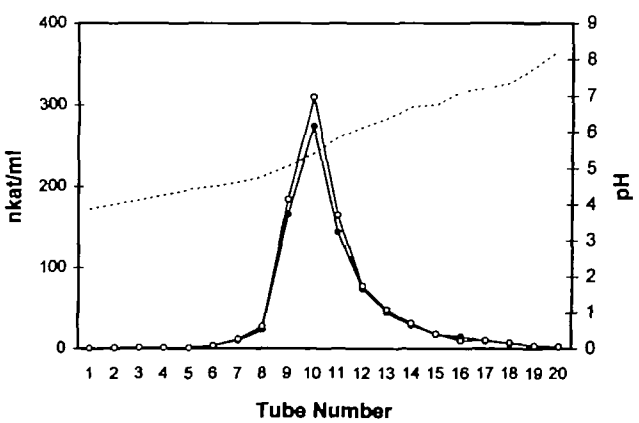

Fig. 1. **Isoelectric focusing of the dialyzed 35-75% ammonium sulfate precipitate from *Dalbergia cochinchinensis* Pierre.** Isoelectric focusing was performed in a Rotofor apparatus using 2% ampholyte (with ratio pH 4-6 : pH 3-10 of 5 : 1) for 4 h at 12 W. Tubes 8-14 were pooled for further purification. ●, β-glucosidase activity (nkat/ml); ○, β-fucosidase activity (nkat/ml); ·····, pH.

subunits in flax (*11*), and variable aggregates of 4, 6, 8, 10, or more subunits in cassava (*26*).

Analysis of purified enzyme by non-denaturing polyacrylamide gel electrophoresis showed a single protein band at the same position as the hydrolytic activity towards 4-MU-β-D-glucoside (Fig. 3B), providing additional evidence for homogeneity. Moreover, the hydrolytic activities toward 4-MU-β-D-fucoside, 4-MU-β-D-galactoside, and 4-MU-α-L-arabinoside were all found to migrate at the same position as the β-glucosidase band (Fig. 4), suggesting that all these substrates are hydrolyzed by the same enzyme molecule.

Kinetic Studies with Purified β-Glucosidase/β-Fucosidase—Optimum pH for hydrolysis of both *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside was pH 5.0 (data not shown), similar to the values of 5.0 to 7.0 observed for many β-glucosidases (*12, 13, 27*). Studies with various *p*-NP-glycosides at 5 mM (Table II) indicated that the enzyme showed by far the highest activity for *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside, similar to that observed in many β-glucosidases (*13, 14, 27, 28*) and in contrast to the "strict" β-fucosidase from *Lactuca sativa* latex (*22*), which has high activity for only *p*-NP-β-D-fucoside and *p*-NP-β-L-fucoside. The Thai Rosewood enzyme had no activity towards *p*-NP-α-D-glycosides, indicating its specificity to the β-glycosidic linkage. However, relative activities in the range of 3-9% of the activity for *p*-NP-β-D-glucoside were observed for the hydrolysis of *p*-NP-β-D-galactoside, *p*-NP-α-L-arabinoside, *p*-NP-β-D-xyloside, and phenyl-β-D-glucoside. This ability to hydrolyze *p*-NP-β-D-galactoside, *p*-NP-α-L-arabinoside, or *p*-NP-β-D-xyloside has been observed in various β-glucosidases (*14, 27*) and β-fucosidases (*21*), and probably results from the fact that these three glycosides have the same *trans*-equatorial configuration with respect to oxygen at C_1, C_2, and C_3 as *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside.

The K_m and k_{cat} values were determined for selected *p*-NP-glycosides (Table III), where the k_{cat} values shown were calculated per subunit rather than per native molecule (which would give 5-fold higher values), since we have greater confidence in the subunit molecular weight obtained by SDS-polyacrylamide gel electrophoresis compared to

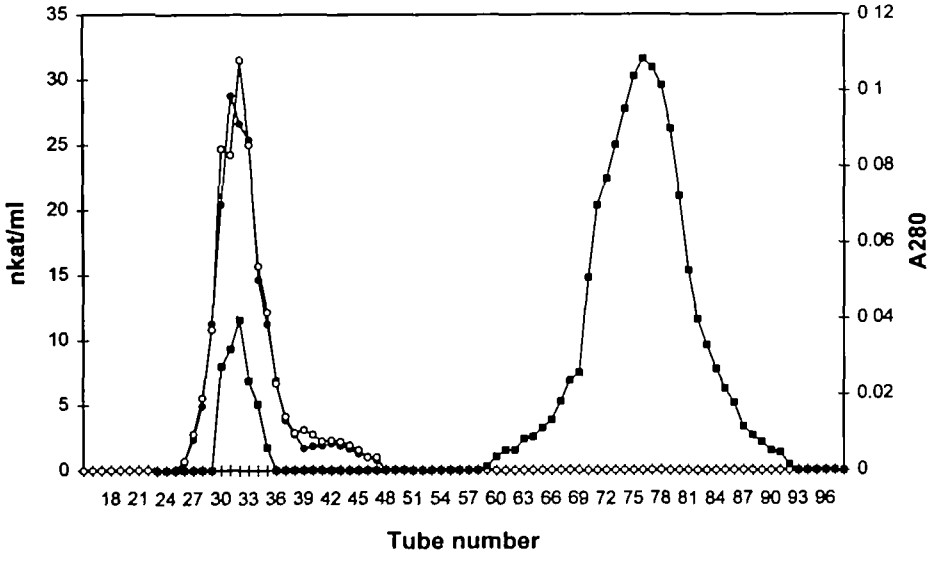

Fig. 2. **Sephadex G-150 chromatography of the isoelectric focusing pool from *Dalbergia cochinchinensis* Pierre.** Enzyme containing fractions from isoelectric focusing (Fig. 1) were concentrated by ultrafiltration and subjected to chromatography in a Sephadex G-150 column (2.5 cm × 80 cm), equilibrated with 0.1 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl at a flow rate of 21 ml/h. Tubes 26-38 pooled, dialyzed, and lyophilized. ●, β-glucosidase activity (nkat/ml); ○, β-fucosidase activity (nkat/ml); ■, A_{280}.

TABLE I. **Purification of β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** Ten grams of seeds were used for purification, and assays were performed with 1 mM *p*-NP-β-D-glucoside or 1 mM *p*-NP-β-D-fucoside at pH 5.0 and 30°C.

Fraction	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract					
β-Glucosidase	2,633	40.5	65.0	1.00	100
β-Fucosidase	3,167	40.5	78.2	1.00	100
35-75% ammonium sulfate					
β-D-Glucosidase	1,750	17.5	100.0	1.54	66.5
β-D-Fucosidase	2,017	17.5	115.3	1.47	63.7
Rotofor isoelectric focusing					
β-D-Glucosidase	1,117	11.4	98.0	1.51	42.4
β-D-Fucosidase	1,333	11.4	116.9	1.49	42.1
Amicon ultrafiltration					
β-D-Glucosidase	1,183	9.45	125.2	1.93	44.9
β-D-Fucosidase	1,383	9.45	146.3	1.87	43.7
Sephadex G-150					
β-D-Glucosidase	800	1.00	800	12.3	30.4
β-D-Fucosidase	1,033	1.00	1,033	13.2	32.6

the native molecular weight obtained by gel filtration. It was interesting to note that the enzyme showed both higher K_m and higher k_{cat} values for *p*-NP-β-D-glucoside compared to *p*-NP-β-D-fucoside. However, *p*-NP-β-D-fucoside had a substantially higher k_{cat}/K_m ratio than any other *p*-NP-glycoside, so it might be argued that the enzyme was a β-fucosidase rather than a β-glucosidase. This high k_{cat}/K_m ratio for the *p*-NP-β-D-fucoside appears to result from a 10-fold lower K_m (0.54 mM) for this substrate compared to the K_m value (5.37 mM) for *p*-NP-β-D-glucoside. This contrasts with many reports, which indicate lower and similar K_m values in the range 0.1-0.5 mM for *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside with β-glucosidase (*14, 15, 28, 29*) and β-fucosidase (*21*).

In addition, the results indicate that the K_m values (1-2.5 mM) for the xyloside and the arabinoside were intermediate between the K_m value for the fucoside and the K_m values for the glucoside and the galactoside. Since the pentosides have a H- atom at position C-5, while the fucoside has a $-CH_3$ group at C-5, and the other hexosides have a $-CH_2OH$ at C-5, it is possible that the presence of a hydrophilic substituent at C-5 detracts from binding efficiency. Even more pronounced differences have been observed in other enzymes, where substantially lower K_m values were found for *p*-NP-α-L-arabinoside and *p*-NP-β-D-xyloside compared to *p*-NP-β-D-glucoside in white clover (*14*) and cassava linamarase (*29*).

Hydrolytic activity towards natural substrates was also tested, for the most part by detecting the release of glucose (Table IV), but rates of hydrolysis were low compared to the hydrolysis of *p*-NP-β-D-glucoside. Hydrolysis of glucose disaccharides was rather slow, with cellobiose being the least readily hydrolyzed disaccharide. Although the enzyme showed little or no hydrolysis of the cyanogenic glycosides linamarin and prunasin, some hydrolysis of amygdalin was observed, but HPLC analysis showed the products were glucose and prunasin (data not shown), indicating cleavage of the β(1-6) bond in the gentiobiose moiety rather than release of cyanide. No cleavage of phloridzin could be observed, but some hydrolysis of salicin was detectable.

Studies of comparative rates of hydrolysis are subject to some variation because the substrates may differ in both K_m and V_{max}, and different results may be obtained at different concentrations of each substrate. Alternatively, comparisons may be made of K_m and V_{max} values, but this requires more detailed studies. For simplicity, we have performed our studies at 5 mM, since we believe that this concentration should be sufficient to detect substrates that are well hydrolyzed *in vivo*, it being 10-fold higher than the K_m of the best substrate (*p*-NP-β-D-fucoside). But rates of

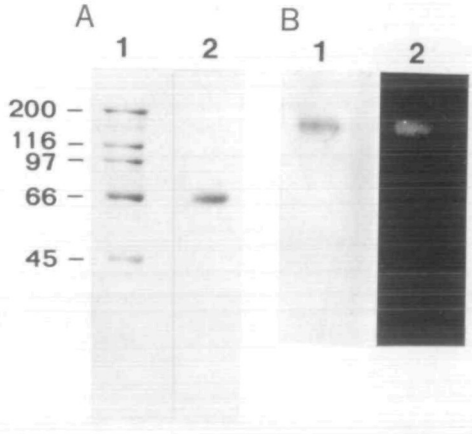

Fig. 3. **Electrophoresis of purified β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** A: SDS-PAGE with protein stain: 1, molecular weight markers, 2, purified enzyme (10 μg). B: Non-denaturing gel of purified enzyme 1, 2 4 μg enzyme stained for protein; 2, 2 4 μg enzyme stained for β-glucosidase activity.

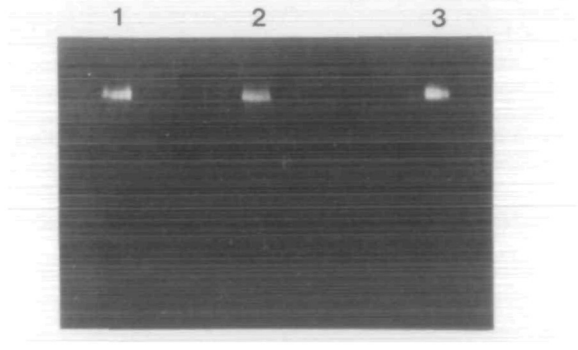

Fig 4. **Non-denaturing electrophoresis of purified β-glucosidase/β-fucosidase followed by activity staining.** 1, 1 2 μg of enzyme stained for 4-MU-β-D-fucoside; 2, 36 μg of enzyme stained for 4-MU-β-D-galactoside, 3, 36 μg of enzyme stained for 4-MU-α-L-arabinoside.

TABLE II. **Hydrolysis of synthetic substrates by β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** Reactions employed 5 mM glycosides in 0.1 M sodium acetate, pH 5.0, at 30°C. *p*-Nitrophenol was quantitated, except with the methyl- and phenyl-glucosides, where glucose was measured.

Substrate	Relative activity
p-NP-β-D-glucoside	100.0
p-NP-β-D-fucoside	124 1
p-NP-β-D-galactoside	8.95
p-NP-β-D-mannoside	0.26
p-NP-β-D-xyloside	3.91
p-NP-β-L-fucoside	0.05
p-NP-α-D-glucoside	0 18
p-NP-α-D-galactoside	0.03
p-NP-α-D-mannoside	0.36
p-NP-α-L-arabinoside	4.89
p-NP-α-L-fucoside	0 08
p-NP-β-D-maltoside	0.21
p-NP-β-D-thioglucoside	0.02
p-NP-β-D-thiofucoside	0.03
Methyl-β-D-glucoside	0.18
Phenyl-β-D-glucoside	5.00

TABLE III. **Kinetic properties of β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** Assays were performed in 0.1 M sodium acetate buffer, pH 5 0, at 30°C. k_{cat} was estimated assuming a subunit molecular weight of 66,000.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
p-NP-β-D-glucoside	5.37±0.09	307 ±4 6	57,300
p-NP-β-D-fucoside	0.54±0.04	151 ±3 0	283,100
p-NP-β-D-galactoside	14.58±0.71	44 ±0 8	3,000
p-NP-α-L-arabinoside	1.00±0 03	6 8±0.04	6,900
p-NP-β-D-xyloside	2.45±0.14	1.8±0.04	730

TABLE IV. **Hydrolysis of natural substrates by β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** Reactions employed 5 mM substrates in 0.1 M sodium acetate buffer, pH 5.0, at 30°C for 30 min, and glucose release was measured. With laminarin (5 mg/ml), reducing equivalents were measured.

Substrate	Relative activity
p-NP-β-D-glucoside (control)	100.0
Sophorose [β1-2]	0.39
Laminaribiose [β1-3]	0.34
Cellobiose [β1-4]	0.06
Gentiobiose [β1-6]	0.29
Linamarin	<0.05
Prunasin	<0.1
Amygdalin	4.55
Salicin	3.75
Phloridzin	<0.05
Arbutin	1.15
Sinigrin	0.86
Laminarin	<0.05

TABLE V. **Effect of various substances on β-glucosidase/β-fucosidase from *Dalbergia cochinchinensis* Pierre.** Substances were tested for their effects on the hydrolytic activity towards *p*-NP-β-D-glucoside (2 mM) or *p*-NP-β-D-fucoside (1 mM) in 0.1 M sodium acetate buffer, pH 5.0 at 30°C.

Substance	Final concentration	% activity remaining	
		β-D-Glucosidase	β-D-Fucosidase
Control	—	100.0	100.0
$FeSO_4$	1 mM	112.9	99.9
$FeCl_3$	1 mM	104.2	113.6
$CaCl_2$	1 mM	102.1	93.8
$MnCl_2$	1 mM	94.8	91.5
KCN	1 mM	106.7	104.5
$ZnSO_4$	1 mM	111.2	107.6
$MgCl_2$	1 mM	111.3	112.1
NaF	1 mM	104.4	91.8
$HgCl_2$	1 mM	0.3	3.1
$HgCl_2$	10 μM	9.2	10.8
$HgCl_2$	0.1 μM	10.7	9.4
p-Chloromercuribenzoate	1 mM	11.7	10.2
p-Chloromercuribenzoate	10 μM	15.2	13.2
p-Chloromercuribenzoate	1 μM	25.2	23.7
EDTA	1 mM	93.9	99.3
Iodoacetate	1 mM	84.2	98.3
δ-Gluconolactone	1 mM	8.8	6.8
δ-Gluconolactone	0.1 mM	50.3	69.8

hydrolysis were rather low and in no case did the rate exceed 5% of the hydrolysis rate of *p*-NP-β-D-glucoside. This would tend to negate the idea that any of the substrates tested was the natural substrate, since the relative rates of hydrolysis of natural substrates by β-glucosidases tend to be much higher (*14-17*). Accordingly, further studies will be required to identify the natural substrate *in vivo*.

The effect of various compounds was tested on the hydrolysis of *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside (Table V). Iodoacetate, EDTA, most metal ions (with the exception of mercuric compounds) had little effect on either β-glucosidase or β-fucosidase activity. On the other hand, δ-gluconolactone showed inhibitory activity at the level of 10^{-3} to 10^{-4} M, in agreement with the possible involvement of a lactone transition state in the mechanism of action of many glycosidases (*30, 31*). However, the strongest inhibition was observed with mercuric compounds, at the level of 10^{-7} M for $HgCl_2$ and 10^{-5} to 10^{-6} M for *p*-chloromercuribenzoate. Mercuric compounds often inhibit enzymes by reaction with sulfhydryl groups, but this may not be the case with this enzyme, since another sulfhydryl reagent, iodoacetate, caused no inhibition. It is therefore possible that the very strong inhibition by mercuric compounds may be due to chelation of catalytically active acidic amino acids in the enzyme, as has been suggested for other enzymes (*32*).

In conclusion, we have purified an enzyme containing hydrolytic activity to both *p*-NP-β-D-glucoside and *p*-NP-β-D-fucoside from the seeds of Thai Rosewood, and characterized various of its properties. However, there are many types of β-glucosidase, most of which also have β-fucosidase activity (*10*). The fact that we are still unable to define the natural substrate of the enzyme, even after testing several compounds, makes it difficult to know which type of β-glucosidase the enzyme is. However, examination of β-glucosidase and β-fucosidase activities in other tissues, including leaf, stem, petiole, and shoot tip, indicated that none of these tissues contained either enzyme activity in excess of 3.5 nkats per g tissue, which is only about 1% of the activity found in the seed. So the major function of the enzyme is likely to involve hydrolysis of substrates in the seed, and attempts are being made to examine the constituents of the seed to find the putative natural substrate. Another approach towards classification of the enzyme would be sequence analysis, since glycosidases can be divided into several groups based on comparison of amino acid sequences (*33, 34*). Studies on the amino acid sequence of the enzyme are also in progress.

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