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Characterization of a Novel Rotenoid-b-Glucosidase Enzyme and its Natural Substrate from Thai Rosewood

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Abstract: A β-glucosidase enzyme was purified from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre) by ammonium sulfate fractionation, preparative isoelectric focusing and Sephadex G-150 chromatography. The electrophoretically homogeneous product had molecular weights of 66,000 in the denatured state and 330,000 in the native state. p-Nitrophenyl-β-D-glucoside (K_m 5.4 mM; k_{cat} 307 sec^{-1}) and p-NP-β-D-fucoside (K_m 0.54 mM; k_{cat} 151 sec^{-1}) were hydrolyzed at the same active site with pH optimum of 5.0. Inhibition was observed with δ-gluconolactone, $HgCl_2$, conduritol B epoxide and Tris. The presumed natural substrate, present in the seed at 3.5% by weight, was purified by silica gel column chromatography and reversed phase TLC., and its structure was determined by NMR. and mass spectroscopy to be a rotenoid β-glucoside. Despite its unusual specificity, sequence analysis indicated that rotenoid β-glucosidase is clearly homologous to other β-glucosidases in its N-terminal sequence. Reversal of the hydrolytic action of Thai Rosewood β-glucosidase by 50% (w/w) D-glucose at 50°C gave disaccharides and trisaccharides in 30% yield, with gentiobiose as the major product. Novel hetero-oligosaccharides could be synthesized from mixtures of D-glucose and D-fucose. Since rotenoids have insecticidal properties, the rotenoid glucoside and its specific β-glucosidase enzyme may play a role in protection against insects.

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*Invited lecture presented at the International Conference on Biodiversity and Bioresources: Conservation and Utilization, 23–27 November 1997, Phuket, Thailand. Other presentations are published in *Pure Appl. Chem.*, Vol. 70, No. 11, 1998.

INTRODUCTION

Glycosidase enzymes (EC 3.2.3.x) catalyze the hydrolysis of glycosidic linkages 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 (2-3), as well as for oligosaccharide synthesis by reversal of their hydrolytic action (4-5). We have therefore screened for nine glycosidase enzymes from more than 50 species of Thai plants using p-NP-glycosides as substrates (6). Very high levels of β-glucosidase and β-fucosidase activities were found in the seeds of *Dalbergia cochinchinensis* Pierre (Thai Rosewood), compared to the levels of these or other glycosidase enzymes in other plant seeds, so more detailed studies were performed on these enzyme activities. The β-glucosidases [EC 3.2.1.21] are a heterogeneous group of enzymes (7) with various functions, such as hydrolysis of cyanogenic glucosides (8-11), glucose disaccharides (12-13), phenolic glycosides (14) or thioglucosides (15), while β-fucosidases [EC 3.2.1.38] are less common and have less well-known functions (16, 17). This paper reviews work performed in our laboratories on the purification, kinetic studies, isolation of substrate, and reverse hydrolysis catalysis of the β-glucosidase/ β-fucosidase from Thai Rosewood.

PURIFICATION OF THAI ROSEWOOD b-GLUCOSIDASE/ b-FUCOSIDASE

β-Glucosidase and β-fucosidase activities were purified from imbibed Thai Rosewood seed extract by 35-75% ammonium sulfate precipitation, preparative isoelectric focusing (pH 4-6), ultrafiltration, and Sephadex G-150 chromatography (18). The fold purification and cumulative yield of the β-glucosidase and β-fucosidase activities were similar at all steps, including the final product, indicating that they co-purify. Analysis of the final product by non-denaturing polyacrylamide gel electrophoresis showed a single band of protein at the same position as the single band of β-glucosidase activity and the single band of β-fucosidase activity, suggesting that a single enzyme is responsible for both β-glucosidase and β-fucosidase activities. The purity of enzyme was confirmed by SDS-polyacrylamide gel electrophoresis, which showed a single band of M_r 66,000 daltons (18). With native enzyme, pI was 5.5 and molecular weight was 330,000 daltons, suggesting that active enzyme consists of 4-6 subunits of 66,000 daltons.

KINETIC PROPERTIES OF b-GLUCOSIDASE/ b-FUCOSIDASE

Our kinetic studies (18) indicate that the purified Thai Rosewood enzyme was able to hydrolyze several p-nitrophenyl(NP)-glycosides at 5 mM, but p-NP-β-D-glucoside and p-NP-β-D-fucoside were by far the best substrates, similar to that observed in many β-glucosidases (8, 9). However, studies with several commercially available natural substrates showed that these compounds were poorly hydrolyzed, including the glucose β-linked disaccharides (sophorose, laminaribiose, cellobiose, gentiobiose), cyanogenic glycosides (linamarin, prunasin, amygdalin), phenolic glycosides (arbutin, salicin, phloridzin) and thioglucoside (sinigrin). The Thai Rosewood enzyme could therefore not be classified into any of the known β-glucosidase groups, such as cyanogenic glucosidases, cellobiase, gentiobiase, phenolic glucosidase, or myrosinase (thioglucosidase).

TABLE 1: Hydrolysis of Some Substrates by Thai Rosewood β-Glucosidase/β-Fucosidase

p-Nitrophenyl glycosides[a]	**Relative activity**	**Naturally occurring substrates**	**Relative activity**
p-NP-β-D-glucoside	100.0	p-NP-β-D-glucoside	100.0
p-NP-β-D-fucoside	124.1	sophorose [β1-2]	0.39
p-NP-β-D-galactoside	8.95	laminaribiose [β1-3]	0.34
p-NP-β-D-mannoside	0.26	cellobiose [β1-4]	0.06
p-NP-β-D-xyloside	3.91	gentiobiose [β1-6]	0.29
p-NP-α-D-glucoside	0.18	linamarin	<0.05
p-NP-α-D-galactoside	0.03	prunasin	<0.1
p-NP-α-D-mannoside	0.36	amygdalin	4.55
p-NP-α-L-arabinoside	4.89	salicin	3.75
p-NP-β-D-maltoside	0.21	phloridzin	<0.05
p-NP-β-D-thioglucoside	0.02	arbutin	1.15
p-NP-α-L-fucoside	0.08	sinigrin	0.86

Reactions employed 5 mM glycosides in 0.1 M sodium acetate, pH 5.0 at 30°C.
[a] p-nitrophenol released in 10 min; [b] glucose released (glucose oxidase kit) in 30 min.

More detailed kinetic studies of the Thai Rosewood enzyme were also performed to determine the K_m and the k_{cat} values of the enzyme for various p-NP-β-D-glycosides (Table 3). Interestingly, the enzyme shows both a higher K_m value and higher k_{cat} value for p-NP-β-D-glucoside than for p-NP-β-D-fucoside. Moreover, the k_{cat}/K_m ratio of the enzyme is highest for p-NP-β-D-fucoside, indicating that this substrate is the most efficiently hydrolyzed. Other p-NP-glycosides are less well hydrolyzed, but 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.

TABLE 2. Kinetic Constants of Thai Rosewood β-Glucosidase/β-Fucosidase

Substrate	**K_m mM**	**k_{cat}* sec^{-1}**	**k_{cat}/K_m $M^{-1}.sec^{-1}$**
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

* k_{cat} was calculated per subunit of M_r 66,000 daltons

The effect of various potentially inhibitory compounds was tested on the Thai Rosewood enzyme (18). No inhibition was observed with $FeCl_3$, $CaCl_2$, KCN, $ZnSO_4$, $MgCl_2$, NaF, EDTA or iodoacetate. 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 some glycosidases (19). However, the strongest inhibition was observed with mercuric compounds, at the level of 10^{-7} M for $HgCl_2$ and at the level of 10^{-5} to 10^{-6} M for p-chloromercuribenzoate p-CMB. Mercuric compounds usually inhibit enzymes by reaction with sulfhydryl groups, but with this enzyme, another sulfhydryl reagent, iodoacetate caused no inhibition. So it is possible that the very strong inhibition by mercuric compounds result from chelation of catalytically active acidic amino acids in the enzyme, as suggested for other enzymes (20).

TABLE 3. Effect of Various Compounds on the Activity of Thai Rosewood β-Glucosidase

Substance	**Final conc**	**% Glucosidase remaining**	**Substance**	**Final conc**	**%Glucosidase remaining**
Control	-	100.0	Control	-	100.0
$FeCl_3$	1 mM	104.2	$HgCl_2$	1mM	0.3
$CaCl_2$	1 mM	102.1	$HgCl_2$	10 μM	9.2
KCN	1 mM	106.7	$HgCl_2$	0.1 μM	10.7
$ZnSO_4$	1 mM	111.2	p-CMB	1 mM	11.7
$MgCl_2$	1 mM	111.3	p-CMB	10 μM	15.2
NaF	1 mM	104.4	p-CMB	1 μM	25.2
EDTA	1 mM	93.9	δ-Gluconolactone	1mM	8.8
Iodoacetate	1 mM	84.2	δ-Gluconolactone	0.1 mM	50.3

Substances were tested on the hydrolysis of 2 mM pNP-β-D-glucoside in 0.1 M sodium acetate, pH 5.0.

SINGLE ACTIVE SITE FOR b-GLUCOSIDASE AND b-FUCOSIDASE

Various studies were performed to determine whether the β-glucosidase and β-fucosidase activities are located at the same active site. First, conduritol B epoxide (CBE), a racemic mixture of 1-L-1,2-anhydro-myo-inositol and 1-D-1,2-anhydro-myo-inositol, inhibited both β-glucosidase and β-fucosidase activities to similar extents, with a pseudo-first-order rate constant (k_{obs}) of inactivation of 5.56 x 10^{-3} sec^{-1}, and binding stoichiometry of 0.9 mol per subunit (21). Partially inactivated enzyme showed similar kinetics with of p-NP-β-D-glucoside and p-NP-β-D-fucoside as substrates, and Tris at 300 mM protected both β-glucosidase and β-fucosidase activities from inactivation by 6 mM CBE (21). p-NP-β-D-Fucoside showed competitive inhibition of p-NP-β-D-glucoside hydrolysis with K_i of 0.42 mM (22). Moreover, hydrolysis of mixtures of p-NP-β-D-glucoside and p-NP-β-D-fucoside at fractional ratios ranging from 0 to 1 showed Lineweaver-Burk plots intermediate between the two extremes, and the apparent K_m and V_{max} values at each fractional ratio showed good correspondence with the theoretical curve

predicted for the existence of a single common active site for the hydrolysis of the two substrates (22).

IDENTIFICATION OF NATURAL SUBSTRATE OF THAI ROSEWOOD ENZYME

Since none of the commercially available natural substrates was well hydrolyzed, we purified the natural substrate of the Thai Rosewood enzyme by extraction of the seeds with absolute ethanol, silica gel column chromatography with absolute ethanol as solvent, and preparative reversed phase C_{18} TLC using 70% aqueous methanol as solvent (23, 24). The structure of the purified compound was identified by ^{1}H-NMR, ^{13}C-NMR, and COSY (H-H correlation, C-H correlation) on a 400 MHz Bruker Aspect 3000 spectrometer, mass spectroscopy on a Finnigan MAT-90 mass spectrometer, and by IR and UV spectroscopy on Perkin-Elmer System 2000 and Lambda 6 spectrometers. The results (24) indicated that the natural substrate found in Thai Rosewood seeds was an isoflavone glycoside, namely 12-dihydroamorphigenin-8'-β-D-glucoside (Fig. 1). Kinetic studies indicate that this substrate is hydrolyzed at a rate equal to 184 % of the hydrolysis rate of p-NP-β-D-glucoside, when both were assayed at 2 mM. Moreover, we have only found the glucoside and not the fucoside, so the Thai Rosewood enzyme should be considered to be 12-dihydroamorphigenin-8'-β-glucosidase.

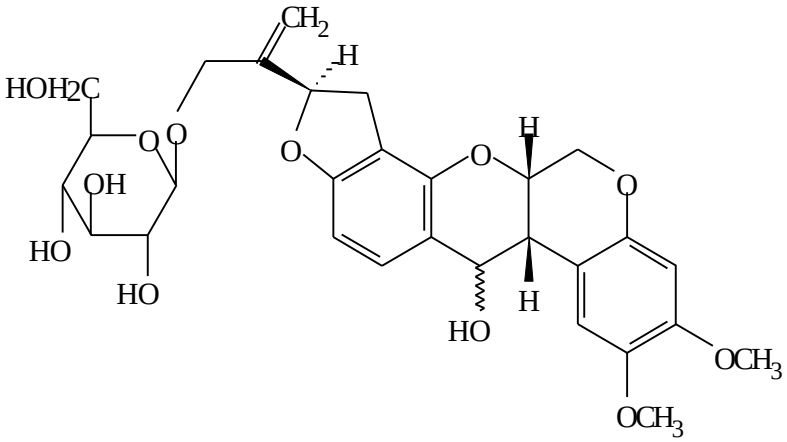

Fig 1. 12-Dihydroamorphigenin-8'-β-D-glucoside, the natural substrate of the enzyme

N-TERMINAL SEQUENCE OF THAI ROSEWOOD b-GLUCOSIDASE

Since 12-dihydroamorphigenin-8'-β-glucosidase is a new enzyme, not previously reported before, the amino acid sequence of the enzyme is being determined. The strategy that we have used is to digest the deglycosylated enzyme with endoproteinase LysC or trypsin, isolate selected peptides by reversed phase HPLC and determine their sequences using a Protein Sequencer (Applied Biosystems Model 473A). Degenerate oligonucleotides were synthesized based on the N-terminal sequence in the forward direction, and internal peptides in both forward and reverse directions. The oligonucleotide primers were used to amplify DNA purified from young *Dalbergia* leaves by PCR, and to amplify RNA purified from leaves and germinated seeds by reverse transcription-PCR (RT-PCR). A specific product of approximately 223 bp was amplified from RNA

preparations was cloned into *E.coli* DH5α using pGEMt vector, and sequenced using an automated DNA sequencer (Applied Biosystems 373A). The sequence obtained (23) was compared to known sequences, retrieved from the NCBI Genbank and SWISSPROT databases through expasy, using the alignment procedure of Feng and Doolittle (25). The results (Fig. 2) indicate that the N-terminal sequence of 12-dihydroamorphigenin-8'-β-glucosidase from *Dalbergia cochinchinensis* (DCBG) shows 37%-51% identity to the other plant β-glucosidases compared.

		Accession
DCBG	= *Dalbergia cochinchinensis* Beta-Glucosidase N-terminal fragment	our data
BCPH	= Black Cherry Prunasin Hydrolase (29-106=78)	1236961
BCAH	= Black Cherry Amygdalin Hydrolase Isoform AH I Precursor (22-99=78).	833835
WCCY	= White Clover Cyanogenic Beta-Glucosidase Precursor (Linamarase) (9-86=78)	114975
CSFG	= *Costus speciosus* Furastanol Glycoside 26-O-Beta-Glucosidase (75-152=78)	1374991
BGQ60	= Barley Beta-Glucosidase Precursor (22-99=78)	1362162
ATT3D	= *Arabidopsis thaliana* Thioglucosidase(EC 3.2.3.1) (19-95=77)	1363489
RPBG	= Rape Beta-Glucosidase (EC 3.2.1.21) (17-93=77)	1076442
CALN	= Cassava Linamarase (Beta-Glucosidase, *Manihot Esculenta*) (12-90=79)	249262
CABG	= Casssava Beta-Glucosidase (23-101=79)	1155090

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DCBG  I D F A K E V R E T I T E V P P F N R S C F P       S D F I F G T
BCPH  T N A A G T Y P P V     V C A T L N R T H F D T L F P G F T F G A
BCAH  S K A A K T D P P I     H C A S L N R S S F D A L E P G F I F G T
WCCY  I H A F K P L P I S F D D F S D L N R S C F A       P G F V F G T
CSFG  T A V E S A T P T A V P S K V V L G R S S F P       R G F I F G A
BGQ60 C D G P N P N P E I   G N T G G L S R Q G F P       A G F V F G T
ATT3D S P T R A E E G P V C P K T E T L S R A S F P       E G F M F G T
RPBG  A S P A N A D G P V C P P S N K L S R A S F P       E G F L F G T
CALN  R P A M G T D D D D D N I P D D F S R K Y F P       D D F I F G T
CABG  L T N Q A T A F D G D F I P L N F S R S Y F P       D D F I F G T

DCBG  A S S S Y Q Y E G         E G R V P S I W D N F T H Q Y P E K I A D R
BCPH  A T A A Y Q L E G A A N I D G R G P S V W D N F T H E H P E K I T D G
BCAH  A S A A Y Q F E G A A K E D G R G P S I W D T Y T H N H S E R I K D G
WCCY  A S S A F Q Y E G A A F E D G K G P S I W D T F T H K Y P E K I K D R
CSFG  A S A A Y Q V E G A W N E G G R G P S I W D T F T H D H P E K I A D H
BGQ60 A A S A Y Q V E G M A R Q G G R G P C I W D A F V   A I Q G M I A G N
RPBG  A T A S Y Q V E G A V N E G C R G P S L W D I Y T K K F P H R V K N H
ATT3D A T A A Y Q V E G A I N E T C R G P A L W D I Y C R R Y P E R C N N D
CALN  A T S A Y Q I E G E A T A K G R A P S V W D I F S K E T P D R I L D G
CABG  A T S A Y Q I E G A A N K F G R G A S V W D T F T H Q Y P E R I L D H

DCBG  S N G D V A V D Q F H R Y
BCPH  S N G D V A I D Q Y H R Y
BCAH  S N G D V A V D Q Y H R Y
WCCY  T N G D V A I D E Y H R Y   Gap penalty = 8, PAM 250 Matrix
CSFG  S N G D K A T D S Y K K Y   Bold italic = identical in all enzymes
BGQ60 G T A D V T V D E Y H R Y
RPBG    N A D V A V D F Y H R F
ATT3D   N G D V A V D F F H R Y
CALN  S N G D V A V D F Y N R Y
CABG  S T G D V A D G F Y Y R F
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Fig. 2. Comparison of N-terminal Sequence of β-Glucosidase from *Dalbergia cochinchinensis*

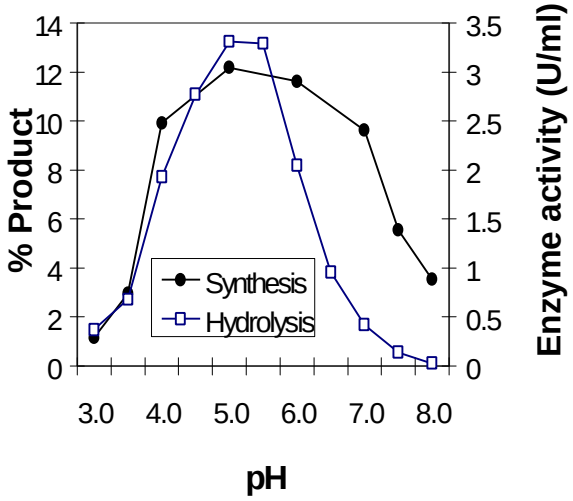
OLIGOSACCHARIDE SYNTHESIS BY REVERSE HYDROLYSIS

Enzymatic reactions are, in principle, reversible. Thus the normal reaction of glycosidase enzymes, where oligosaccharides are hydrolyzed to smaller compounds, disaccharides and monosaccharides may potentially be reversed under suitable conditions to achieve net synthesis of oligosaccharides, as follows.

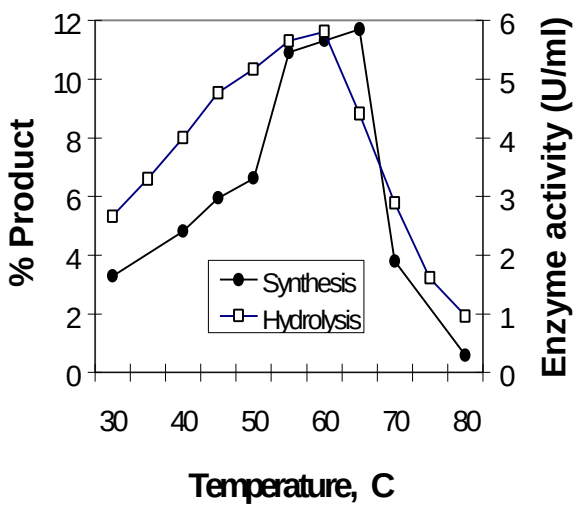
$$\text{Monosaccharide} \xleftarrow{-H_2O} \text{Disaccharide} \xleftarrow{-H_2O} \text{Higher oligosaccharide}$$

Our studies of oligosaccharide synthesis by reverse hydrolysis have involved equilibrium synthesis, where enzyme is incubated with high concentrations of monosaccharide and low water activity at elevated temperature for prolonged periods, which allow an approach to equilibrium. When Thai Rosewood β-glucosidase was incubated with 50% (w/w) D-glucose as substrate for 7 days at 50°C, analysis of the products by Aminex HPX-87C HPLC (Waters 625 LC) using refractive index detection showed the presence of disaccharides and trisaccharides with total combined yields of 15-30% (26). Improved resolution of disaccharides could be achieved by anion exchange HPLC on a Dionex CarboPac PA-1 column with pulsed amperometric detection, where the glucose disaccharides, gentiobiose [β1-6], cellobiose [β1-4], lamaribiose [β1-3] and sophorose [β1-2] could be separated. Analysis of the products of synthesis with 50% (w/w) D-glucose on the Dionex column showed that gentiobiose was the major product of the synthesis reaction, with the ratio of gentiobiose: laminaribiose plus sophorose: cellobiose being about 3:1:0.

A. pH effect



B. Temperature



C. Glucose concentration (% w/w) *D. Enzyme Amount (Units)*

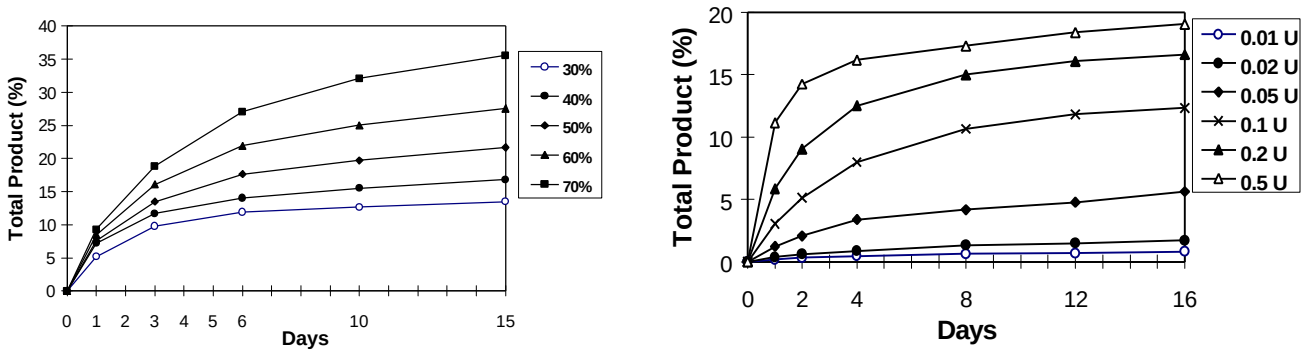


Fig. 3. Effect of varying conditions of oligosaccharide synthesis by Thai Rosewood β-glucosidase

The optimum pH and optimum temperature of the Thai Rosewood β-glucosidase/β-fucosidase (26) were the same for the hydrolysis and for the synthesis reaction, namely pH 5.0 and 60-65°C (Fig. 3A and 3B). Increasing the amount of enzyme or substrate in the reaction mixture (26) also increased the amount of products formed (Fig, 3C and 3D). With low levels of enzyme, little product was formed, and increasing the amount of enzyme seemed to affect not only the rate at which equilibrium is approached, but also the amount of product formed at the approach of equilibrium. Increasing the concentration of glucose tended to increase the amount of product formed, presumably due to a mass action effect. High monosaccharide concentrations not only shift the equilibrium towards synthesis because of the monosaccharide concentration *per se*, but they also lower water activity, because in a 70% (w/w) monosaccharide solution, the molar ratio of monosaccharide to water is about 0.23, and bearing in mind that monosaccharides have 5 -OH groups, there will be more -OH groups from sugar than from water.

Initially, it seemed surprising to us that enzyme continued to be active for extended periods of 1 week or more at the elevated temperature. Thus, to test the stability of the enzyme at 60°C, a time course study of enzyme activity was performed in the absence and presence of sugar, with synthesis also being followed in the latter. Interestingly, more than 50% of the activity of the enzyme was retained after 7 days at 60°C, at which time equilibrium is being approached. However, when enzyme is incubated at 60°C at the same pH, but in the absence of the glucose substrate, more than 85% of the enzyme activity is lost within 8 hours. Thus high concentrations of sugar also help to stabilize the enzyme and allow higher reaction temperatures to be used for prolonged times, so that equilibrium can be reached.

Synthesis with other monosaccharides was also tested, but little synthesis was observed even with D-fucose, unlike in the hydrolysis reaction, where p-NP-β-D-fucoside had a higher k_{cat}/K_m ratio than p-NP-β-D-glucoside. However, novel synthesis product(s), presumed to be hetero-oligosaccharides could be detected when mixtures of D-glucose and D-fucose were used. The levels of hetero-oligosaccharide reached a maximal yield of 5% when a mixture of 25% w/w glucose: 25% w/w fucose was used. A major product has now been purified, and preliminary studies by NMR spectroscopy suggest that the compound is D-fucosyl(β1-6)-D-glucose.

CONCLUSIONS

Initially, the β-glucosidase enzyme from the seeds of Thai Rosewood (*Dalbergia cochinchinensis* Pierre) was discovered by screening studies, and purified based on its ability to hydrolyze synthetic substrates. The substrate 12-dihydroamorphigenin-8'-β-glucoside, is a novel isoflavone glycoside, not previously described from natural sources, while a specific β-glucosidase for this substrate is also a novel finding. Most interestingly, the substrate is present at very high levels of about 3.55% by weight in the seed, similar to the levels found for amorphigenin-8'-O-β-D-glucoside and 12a-hydroxyamorphigenin-8'-O-β-D-glucoside in the seeds of *Dalbergia monetaria* (27), and much higher that the levels of isoflavonoid O-glycosides found in *Dalbergia latifolia* (28), *Dalbergia nitidula* Welw. ex. Bak.(29), and *Dalbergia paniculata* (30). In addition, the specific 12-dihydroamorphigenin-8'-β-glucosidase enzyme is also present at high levels of 3 mg per 10 g seeds. The high levels of both enzyme and substrate in the seed suggest that both play important biological roles, for example as a protection mechanism against insects. Thus, the aglycone 12-dihydroamorphigenin may be toxic to insects like other isoflavonoids (31), while its glucoside may not be toxic, in which case, ingestion of the less toxic 12-dihydroamorphigenin-8'-β-D-glucoside together with its β-glucosidase enzyme will lead to release of the more toxic aglycone in the insect. This needs to be confirmed by measurement of insecticidal activity of both glucoside and aglycone.

Despite its novel substrate specificity, *Dalbergia cochinchinensis* β-glucosidase shows homology with other β-glucosidases in the glycosyl hydrolase group I described by Henrissat (32). The sequence similarity is only moderate, ranging from 37%-51%, but the N-terminal sequences of the glycosyl hyrolases tend to be more variable. Moreover inhibition by conduritol B epoxide suggests the involvement of acidic amino acid(s) in catalysis, similar to other glycosidases, and this is in agreement with the pH optimum of the enzyme at pH 5.0, and the strong inhibition observed with mercuric compounds and the lack of inhibition by iodoacetate.

The hydrolytic action of *Dalbergia cochinchinensis* β-glucosidase can be reversed leading to synthesis of oligosaccharides, and novel glucosyl-fucose hetero-oligosaccharides may be synthesised. However, given its unusual substrate specificity, the enzyme may be more usefully applied to synthesise glycosides of isoflavonoids, flavonoids and steroids. Additionally, the enzyme might be used to screen for isoflavonoid, flavonoid and steroid glycosides in natural sources, so as to isolate compounds of biological value.

ACKNOWLEDGMENT

This work was supported by a grant from the Chulabhorn Research Institute.

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