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# Purification of an isoflavonoid 7-O-β-apiosyl-glucoside β-glycosidase and its substrates from *Dalbergia nigrescens* Kurz

Phimonphan Chuankhayan [a], Yanling Hua [b], Jisnuson Svasti [c], Santi Sakdarat [a], Patrick A. Sullivan [d], James R. Ketudat Cairns [a,*]

[a] *Institute of Science, Suranaree University of Technology, Schools of Biochemistry and Chemistry, 111 University Avenue, Nakhon Ratchasima 30000, Thailand*
[b] *Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[c] *Center for Excellence in Protein Structure and Function, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[d] *Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand*

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**Abstract**

A β-glycosidase was purified from the seeds of *Dalbergia nigescens* Kurz based on its ability to hydrolyse *p*-nitrophenyl β-glucoside and β-fucoside. This enzyme did not hydrolyze various glycosidic substrates efficiently, so it was used to identify its own natural substrates. Two substrates were identified, isolated and their structures determined as: compound **1**, dalpatein 7-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside and compound **2**, 6,2′,4′,5′-tetramethoxy-7-hydroxy-7-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside (dalnigrein7-O-β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside). The β-glycosidase removes the sugar from these glycosides as a disaccharide, despite its initial identification as a β-glucosidase and β-fucosidase.


*Keywords:* *Dalbergia nigrescens* Kurz; Leguminosae; β-Glucosidase; β-Glycosidase substrate; Isoflavonoid glycoside; Apiosyl glucoside

## 1. Introduction

β-glucosidases (3.2.1.21) hydrolyze glycosidic bonds between non-reducing terminal glucose residues and alcohols of various structures. The aglycones released by plant β-glucosidases that have been identified include saccharides, hydroxynitriles, hydroxamic acids, naphthoquinones, alkaloids, and isoflavonoids, among others (Esen, 1993; Poulton, 1990; Babcock and Esen, 1994; Duroux et al., 1998; Warzecha et al., 2000; Svasti et al., 1999). Since some plant β-glucosidases are able to catalyze the release of sugars other than glucose, all plant β-O-linked glycosides are potential β-glucosidase substrates.

Many isoflavonoid glycosides have been isolated from tropical trees of the genus *Dalbergia*, family leguminosae. These include rotenoid derivatives (Chibber and Khera, 1979; Svasti et al., 1999), and other isoflavone derivatives (Mathias et al., 1998; Rajasekhara Rao and Srinivasa Rao, 1991; Sharma et al., 1980; Farag et al., 1999). These compounds include both O-linked and C-linked glycosides, simple glucosides and oligosaccharide glycosides.

Previously, Srisomsap et al. (1996) described the purification and characterization of a β-glucosidase from *Dalbergia cochinchinesis* Pierre seeds and Svasti et al. (1999) described the use of this β-glucosidase to identify its substrate, dalcochinin-12-O-β-D-glucoside from the

* Corresponding author. Tel: +66 44 224304; fax: +66 224185.
*E-mail address:* cairns@ccs.sut.ac.th (J.R. Ketudat Cairns).

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same seeds. Here, we have used the same approach to identify a β-glycosidase with distinct specificity and its substrates and products from the seed of *Dalbergia nigrescens* Kurz.

## 2. Results and discussion

In preliminary experiments, β-glucosidase from *D. cochinchinesis* was shown to hydrolyze a glycoside from *D. nigrescens* seed extract, but not as well as crude *D. nigrescens* β-glucosidase, as judged by the shift in mobility of a spot on TLC. Therefore, the *D. nigrescens* β-glucosidase was purified and used to identify its substrates.

### 2.1. Purification of β-glucosidase/β-fucosidase from D. nigrescens Kurz seeds

The enzyme was extracted from seeds and purified by 35–75% ammonium sulfate fractionation, DEAE anion exchange chromatography and S-300 gel filtration chromatography, as described in the methods. Both *p*-nitrophenol (*p*NP)-β-D-glucoside and *p*NP-β-D-fucoside hydrolysis activities were determined during the purification. The yields for β-glucosidase and β-fucosidase were similar, but the total and specific activities of β-fucosidase were twice as much as those of β-glucosidase at each step (Table 1). The enzyme was purified 33 and 49 fold over the crude extract for β-glucosidase and β-fucosidase, respectively, with 2–3% yield of both total activities, or about 2 mg of enzyme per 50 g of seed. Large amounts of black polyphenolic compounds were found in the seeds, which required extraction in reducing agents and were not completely eliminated until after the DEAE chromatography. Removal of these polyphenolic compounds may partially account for the relatively low protein yields obtained.

The purified enzyme gave a single band with apparent molecular weight of 62–63 kDa on SDS–PAGE (Fig. 1). Gel filtration of the native enzyme on Sephacryl S-300 gave a molecular weight of approximately 240 kDa, so the native enzyme appears to consist of 4 subunits. Non-denaturing polyacrylamide gel electrophoresis of the purified enzyme followed by activity staining with 4-methylumbelliferyl (4-MU) substrates, 4-MU-β-D-glucoside, 4-MU-β-D-fucoside and 4-MU-β-D-galactoside, showed a single fluorogenic product band for each substrate at the same position as the main protein band (Fig. 2). This data suggests that the purified enzyme consists of a single component, which cleaves β-glucosides, β-fucosides, and β-galactosides.

Sequencing of the N-terminus of the purified protein gave the sequence ATITEV in rather low yield. The last 5 residues of this sequence matches residues 10–14 of *D. cochinchinensis* β-glucosidase (Ketudat Cairns et al.,

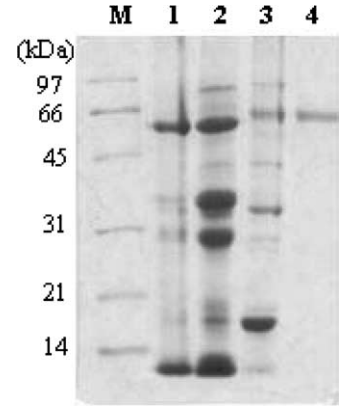



Fig. 1. SDS–PAGE analysis of purified β-glucosidase/β-fucosidase enzyme from *D. nigrescens* seeds. Lane M, Low-range protein markers; 1, crude extract; 2, 35–75% $(NH_4)_2SO_4$ fractionated; 3, DEAE pooled fractions; 4, S 300 gel filtration fraction.

Table 1
Purification of β-glucosidase/β-fucosidase enzyme from *Dalbergia nigrescens* Kurz

| Fraction | Total activity (nkat) | Total protein (mg) | Specific activity (nkat/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | | 3040 | | | |
| β-Glucosidase | 6130 | | 2.02 | 1.00 | 100 |
| β-Fucosidase | 9470 | | 3.11 | 1.00 | 100 |
| 35–75% $(NH_4)_2SO_4$ | | 1390 | | | |
| β-Glucosidase | 4220 | | 3.04 | 1.50 | 68.9 |
| β-Fucosidase | 8680 | | 6.26 | 2.01 | 91.6 |
| DEAE | | 12.3 | | | |
| β-Glucosidase | 574 | | 46.8 | 23.1 | 9.37 |
| β-Fucosidase | 1010 | | 82.2 | 26.4 | 10.6 |
| S300 gel filtration | | 1.79 | | | |
| β-Glucosidase | 119 | | 66.6 | 33.0 | 1.94 |
| β-Fucosidase | 271 | | 152 | 48.9 | 2.86 |

Fifty grams of seeds were used in purification. Enzyme reactions were performed with 1 mM *p*NP−β-D-glucoside or 1 mM *p*NP−β-D-fucoside in 0.1 M sodium acetate, pH 5.0 at 30 °C for 10 min.

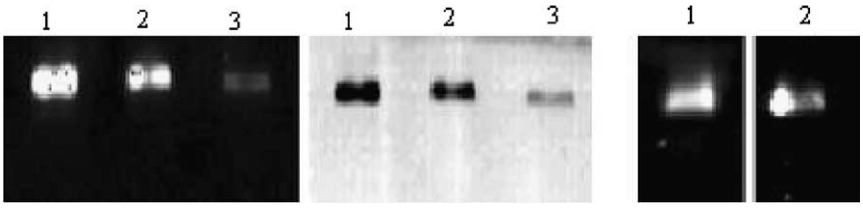



Fig. 2. Activity staining of non-denaturing polyacrylamide gel electrophoresis of *D. nigrescens* β-glucosidase/β-fucosidase enzyme: (a) activity staining with 1 mM of 4-MU-β-D-glucoside; lane 1, 4.8 μg; lane 2, 2.4 μg; lane 3, 1.2 μg of enzyme; (b) protein stained with Coomassie blue R-250; lane 1, 4.8 μg; lane 2, 2.4 μg; lane 3, 1.2 μg of enzyme. C. 10 μg of enzyme stained with 1 mM 4-MU-β-D-fucoside (1) and 1 mM 4-MU-β-D-galactoside (2).

2000). Four tryptic peptides were also sequenced, and they also had high similarity to the sequence of *D. cochinchinensis* β-glucosidase, as shown in Table 2. The sixth residue of the Tryp 2 peptide gave only a low yield of the Ile from the preceding step, which, together with its location 2 residues before Thr and the fact the corresponding residue in *D. cochinchinensis* is Asn, suggests it may be an N-linked glycosylation site. In total, the *D. nigrescens* β-glycosidase was identical with *D. cochinchinensis* β-glucosidase at 51 of 60 residues sequenced, which indicates this enzyme is also a family 1 glycosyl hydrolase (Henrissat, 1991).

### 2.2. Catalytic activity of purified β-glucosidase/β-jucosidase

The enzyme was found to have a pH optimum of 5.0–6.0 with ca half maximal activity at pH 3.5 and 6.5. In the standard 10 min. assay with 1 mM *p*NP-glucoside and *p*NP-fucoside, the temperature optimum was 65 °C. To obtain a preliminary assessment of sugar specificity, *p*NP-glycosides were tested as substrates at 5 mM (Table 3). The enzyme showed the highest activity toward *p*NP-β-D-fucoside and *p*NP-β-D-glucoside, which is similar to *D. cochinchinensis* β-glucosidase (Srisomsap et al., 1996). Other *p*NP-glycosides were hydrolyzed at less than 10% of the rate of *p*NP-β-D-glucoside. The enzyme could not significantly hydrolyze *p*NP-β-cellobioside.

Release of glucose from available natural glycosides and oligosaccharides was also tested. The rates of hydrolysis were extremely low with laminaribiose hydrolyzed at 1.0%, linamarin at 0.37% and cellobiose at 0.33% the rate of *p*NP-β-D-glucoside hydrolysis. No hydrolysis of laminaritriose, cellotriose, salicin, dhurrin, DIMBOA glucoside, sophorose, torvoside A and amygdalin could be observed. The enzyme did cleave dalcochinin-8′-O-β-D-glucoside, the natural substrate of *D. cochinchinensis* β-glucosidase, as judged by TLC analysis (data not shown). However, the activity of the *D. nigrescens* enzyme toward this substrate was rather low compared to that of *D. cochinchinensis* β-glucosidase.

The effect of various compounds was tested on the hydrolysis of *p*NP-β-D-glucoside and *p*NP-β-D-fucoside by the purified *D. nigrescens* glycosidase. Neither the metals tested, $FeSO_4$, $CaCl_2$, $HgCl_2$, $MnCl_2$, and

Table 2
Peptide sequences from *D. nigrescens* β-glycosidase

| Peptide | Sequence |
|---|---|
| N-terminus | |
| *D. nigrescens* | ATITEV |
| *D. cochinchinensis* 9–14 | ETITEV |
| Tryp 1 | |
| *D. nigrescens* | YMNLDAYR |
| *D. cochinchinensis* 82–90 | DMNLDAYR |
| Tryp 2 | |
| *D. nigrescens* | ASGGIISTGVD |
| *D. cochinchinensis* 104–114 | VSGGINQTGVD |
| Tryp 3 | |
| *D. nigrescens* | LINETLANGI |
| *D. cochinchinensis* 119–128 | LINESLANGI |
| Tryp 4 | |
| *D. nigrescens* | HWITVNEPSIFTMNGYAYGIFAPGR |
| *D. cochinchinensis* 176–200 | HWITLNEPSIFTANGYAYGMFAPGR |

The N-terminus and tryptic peptides of the *D. nigrescens* β-glycosidase are shown above the corresponding sequence from the *D. cochinchinensis* β-glucosidase numbered according to its position in the mature protein.

Table 3
Comparison of hydrolysis of synthetic substrates by *D. nigrescens* and *D. cochinchinensis* β-glucosidases

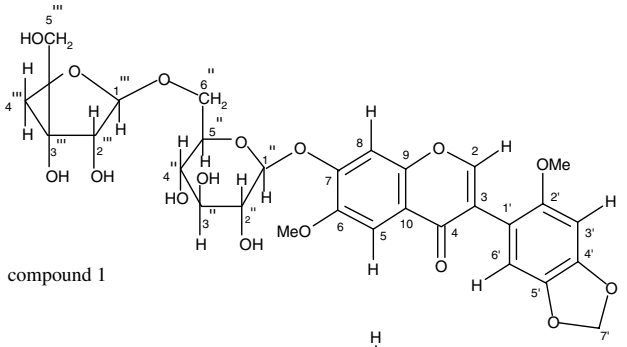
| Substrate | *D. nigrescens* % Relative activity | *D. cochinchinensis* % Relative activity |
|---|---|---|
| *p*NP−β-D-glucoside | 100 | 100 |
| *p*NP−β-D-fucoside | 124 | 124 |
| *p*NP−β-D-galactoside | 3.97 | 8.95 |
| *p*NP−β-D-xyloside | 7.55 | 3.91 |
| *p*NP−α-L-arabinoside | 1.91 | 4.89 |
| *p*NP−β-D-thioglucoside | 0.68 | 0.02 |
| *p*NP−β-L-arabinoside | 0.47 | Not determined |
| *p*NP−β-D-mannoside | 0.40 | 0.26 |
| *p*NP−β-cellobioside | Not detectable | Not determined |

Enzyme reactions were performed with 5 mM *p*NP-glycosides, as described in the methods. Relative activities for *D. cochinchinensis* β-glucosidase are taken from Srisomsap et al. (1996).

$ZnSO_4$, nor EDTA had a significant effect on either β-glucosidase or β-fucosidase activities at 1 mM concentration. In contrast, $HgCl_2$ had a strong inhibitory effect on *D. cochinchinensis* β-glucosidase and garbonzo bean isoflavonoid β-glucosidases (Srisomsap et al., 1996, Hösel and Barz, 1975). Inhibition by 1 mM δ-gluconolactone was also weaker than its effect on *D. cochinchinensis* β-glucosidase, with only 34% inhibition of β-glucosidase activity.

### 2.3. Identification of natural substrates and products

Hydrolysis of methanolic extracts of *D. nigrescens* seeds with the purified enzyme resulted in a shift in a few fluorescent and UV-absorbing spots on analytical TLC. Fractionation of the extract by LH-20 gel filtration chromatography, followed by preparative TLC allowed the two major substrates to be purified and characterized. Another lower abundance substrate, which was seen as a blue fluorescent spot just ahead of the compound **1** substrate on TLC, coeluted with compound **1** on LH-20. This compound also coeluted with compound **1** in reverse-phase HPLC. In order to purify compound **1**, this substrate was digested with *D. cochinchinensis* β-glucosidase, which rapidly digested it, but not compound **1**. This digestion allowed the purification of compound **1**, but not the minor substrate or its aglycone, due to their low abundance. It is possible that other low abundance glycoside substrates were present, but compounds **1** and **2** appeared to be the most abundant substrates.




**Compound 1**

Compound **1** glycoside was obtained as a pale yellow powder. The UV spectrum showed the characteristic absorption of an isoflavone compound ($\lambda_{max}^{MeOH}$260 and 315 nm). From FTMS, this compound showed a molecular related ion peak at *m*/*z* 659 $[M + Na]^+$, which corresponded to the molecular formula $C_{29}O_{16}H_{32}$ and to the 29 peaks in the $^{13}C$ NMR spectrum. Future fragmentation of this signal gave an ion at *m*/*z* 365.

The formula $C_{18}H_{13}O_7$ of the aglycone is consistent with an isoflavone ($C_6$–$C_3$–$C_6$) which contains two methoxy groups and one O–$CH_2$–O at C-7′. The 294 amu derived from mass subtraction indicated 2 sugar rings of five and six carbon, which corresponded to the 11 carbon signals obtained by subtraction of the aglycone carbon signals from the total number of glycoside $^{13}C$ signals observed in the $^{13}C$ NMR (Table 4). After hydrolysis with enzyme or with acid, the spectrum of purified aglycone, compound **1a**, lacked these signals. The HMBC spectrum of compound **1** revealed cross peaks between two methoxy methyl protons at δ 3.66 and δ 3.93 with the C-6 aromatic carbon position at δ 147.31 and C-2′ position at δ 152.77. The $^1H$ NMR spectrum showed five singlet signals at δ 8.19,δ 7.44,δ 7.32,δ 6.87, and δ 6.83, which are attributable to hydrogens at carbons C-2, C-5, C-8, C-3′ and C-6′ of an isoflavone derivative, as showed in Table 1. The COSY experiment showed no proton cross peak, which confirmed the para position of the protons at C-8 and C-5. The $^1H$ NMR signals for the aglycone in DMSO-$d_6$ matched those previously reported for dalpatein from *Dalbergia paniculata* (Adinarayana and Rajasekhara Rao, 1975; Tahara et al., 1993; Radhakrishniah, 1973).

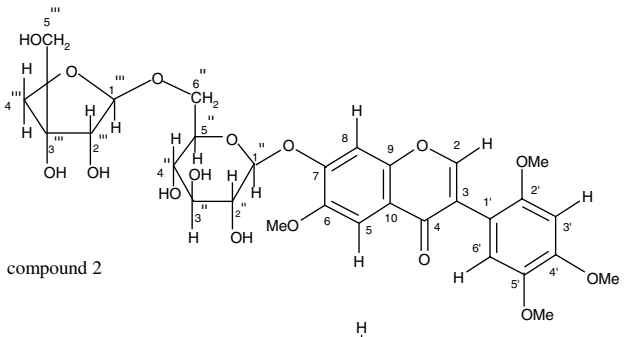
A β-D-apiofuranoside moiety was determined by chemical shift at C-1‴ (δ 109.48), which is ascribable to the anomeric dioxymethine carbon on an apiose ring, along with the carbonic signals of one quaternary carbon at C-3‴ (δ 78.60), two oxymethylenes at C-4‴ (δ 73.30) and C-5‴ (δ 63.32), and one oxymethine at C-2‴ (δ 72.98). The remaining $^{13}C$ signals which corresponded to carbonic carbon were used to elucidate a β-D-glucopyranoside moiety with one anomeric dioxymethine at C-1‴ (δ 99.90), four monooxymethine at C-2‴ (δ 72.98), C-3‴ (δ 76.67), C-4‴ (δ 69.80) and C-5‴ (δ 75.65) carbons. The chemical shift at C-6‴ (δ 67.85) was used to define the disaccharide linkage as apiofuranosyl (C-1‴ → C-6‴) because the chemical shift of a monosaccharide oxymethylene group appears at about $\delta_C$62 (Mathias et al., 1998). Also, the chemical shift at C-1‴ (δ 109.48) indicated C-1 anomeric orientation of apiose as β-configuration because the $^{13}C$ NMR of α-D-apiose would appear at about $\delta_C$104.5 (Takahashi et al., 2001). The NMR spectral data of the sugar part are in agreement with those of tectorigenin 7-O-[β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside] from *Dalbergia sissoo* (Farag et al., 1999).

The aglycone of compound **1** (compound **la**), 6,2′-dimethoxy-4′,5′-methylenedioxyisoflavone, was the same

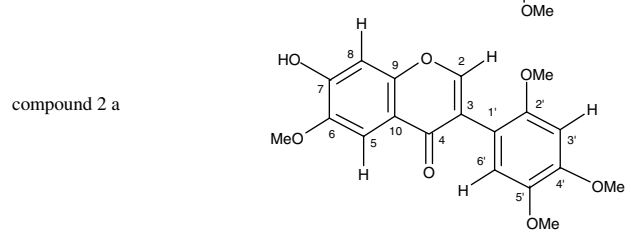
Table 4
$^{1}H$ and $^{13}C$ NMR spectral data of compounds **1**, **2** and **2a**

| Position | Compound **1** (DMSO-$d_6$) | | | Compound **2** ($D_2O$) | | | Compound **2a** ($CD_3OD$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $^{13}C$ | $^{1}H$ | HMBC | $^{13}C$ | $^{1}H$ | HMBC | $^{13}C$ | $^{1}H$ | HMBC |
| 2 | 154.42 CH | 8.19(s) | | 156.40 CH | 8.03(s) | | 156.24 CH | 7.87(s) | |
| 3 | 121.03 C | | 2,3′,6′ | 121.05 C | | 2,3′,6′ | 120.52 C | | 2,3′,6′ |
| 4 | 174.66 C | | 2,5,8 | 177.47 C | | 2,5,8 | 176.48 C | | 2,5,8 |
| 5 | 104.63 CH | 7.44(s) | 8 | 105.21 CH | 7.17(s) | 8 | 102.72 CH | 6.50(s) | 8 |
| 6 | 147.31 C | | 5,8,OMe | 151.43 C | | 5,8,OMe | 152.92 C | | 5,8,OMe |
| 7 | 151.41 C | | 5,8 | 152.29 C | | 5,8 | 153.30 C | | 5,8 |
| 8 | 103.72 CH | 7.32(s) | 5 | 104.10 CH | 7.35(s) | 5 | 101.97 CH | 7.33(s) | 5 |
| 9 | 151.19 C | | 2,5,8 | 147.58 C | | 2,5,8 | 150.18C | | 2,5,8 |
| 10 | 117.68 C | | 2,5,8 | 111.92 C | | 2,5,8 | 111.02 C | | 2,5,8 |
| 1′ | 112.92 C | | 2,3′,6′ | 115.60 C | | 2,3′,6′ | 113.95 C | | 2,3′,6′ |
| 2′ | 152.77 C | | 3′,6′,OMe | 152.16 C | | 3′,6′,OMe | 153.30 C | | 3′,6′,OMe |
| 3′ | 95.4 CH | 6.87(s) | 6′ | 98.72 CH | 6.71(s) | 6′ | 98.76 CH | 6.73(s) | 6′ |
| 4′ | 147.79 C | | 3′,6′,7′ | 149.75 C | | 3′.6′,OMe | 152.06 C | | 3′,6′,OMe |
| 5′ | 140.26 C | | 3′,6′,7′ | 142.35 C | | 3′,6′,OMe | 143.10 CH | | 3′,6′,OMe |
| 6′ | 110.90 CH | 6.83(s) | 3′ | 118.11 CH | 6.86(s) | 3′ | 116.90 CH | 693(s) | 3′ |
| 7′ | 101.08 CH | 6.0(s) | | | | | | | |
| 1″ | 99.90 CH | 5.08(d)$J = 6.84$ | | 100.26 CH | 5.21(d) $J = 7.00$ | | | | |
| 2″ | 72.98 CH | 3.33 | | 72.94 CH | 3.65 | | | | |
| 3″ | 76.67 CH | 3.34 | | 77.09 CH | 3.86 | | | | |
| 4″ | 69.80 CH | 3.15 | | 69.57 CH | 3.59 | | | | |
| 5″ | 75.65 CH | 3.60 | | 75.41 CH | 3.76 | | | | |
| 6″ | 67.85 $CH_2$ | 3.88(*d*)$J = 9.19$ | | 67.76 $CH_2$ | 4.02(d)$J = 9.15$ | | | | |
| 1‴ | 109.48 CH | 4.80(*d*)$J = 2.94$ | | 109.38 CH | 5.02(d)$J = 2.87$ | | | | |
| 2‴ | 75.85 CH | 3.77 | | 75.69 CH | 3.69 | | | | |
| 3‴ | 78.60 C | | | 79.51 C | | | | | |
| 4‴ | 73.30 $CH_2$ | 3.93 | | 73.87 $CH_2$ | 3.92 | | | | |
| 5‴ | 63.32 $CH_2$ | 3.37 | | 64.02 $CH_2$ | 3.53 | | | | |
| 6-OMe | 55.75 | 3.93 | | 6-OMe 56.67 | 3.73 | | 6-OMe 55.98 | 3.80 | |
| 2′-OMe | 56.63 | 3.66 | | 2′-OMe 56.85 | 3.68 | | 2′-OMe 56.35 | 3.75 | |
| | | | | 4′-OMe 56.17 | 3.85 | | 4′-OMe 54.68 | 3.95 | |
| | | | | 5′-OMe 56.38 | 3.82 | | 5′-OMe 55.65 | 3.90 | |

as dalpatein which has been isolated from *D. paniculata* and *Piscidia erythrina*. But compound **1** contained a disaccharide moiety, where as previously only the glucoside (dalpatin) has been identified (Adinaraya and Rajasekhara Rao, 1972).



compound 2



compound 2 a

**Compound 2**

Compound **2** glycoside was also obtained as a pale yellow powder and had a molecular related ion peak $[M + Na]^+$ at *m*/*z* 675 in MS, which corresponded to the molecular formula $C_{30}O_{16}H_{36}$. The future fragmentation of this signal gave an ion at *m*/*z* 381. The UV spectrum showed the characteristic absorption of an isoflavone compound ($\lambda_{max}^{MeOH}$ 260 and 315 nm). The $^{1}H$ NMR spectrum revealed 5 singlet signals at $\delta$ 7.93,$\delta$ 7.39,$\delta$ 6.58,$\delta$ 6.80 and $\delta$ 6.96 attributable to hydrogen at carbons C-2, C-5, C-8, C-3′ and C-6′ of an isoflavone derivative. The gHMBC spectrum had cross peaks between 4 methoxy proton groups which had signals at C-2′ ($\delta$ 3.68(s)), C-6 ($\delta$ 3.71(s)), C-4′($\delta$ 3.85(s)) and C-5′($\delta$ 3.82) and carbon at C-2′ ($\delta$ 152.16), C-6 ($\delta$ 151.43), C-4′ ($\delta$ 149.75) and C-5′ ($\delta$ 142.35). The 11 carbon signals in the region of sugar corresponded to one pentose and one hexose sugar ring, with nearly the same spectrum as in compound **1**, though the compound **2** structure was solved in $D_2O$ rather than DMSO-$d_6$ as solvent. From this comparison, the two compounds appeared to have the same sugar moiety structure. Again, these sugars were removed by hydrolysis with *D. nigrescens* β-glucosidase to give compound **2a**. NMR of compound **2a** was done in $CD_3OD$ because it was less soluble in

$D_2O$ than compound **2**. The $^1H$ NMR spectrum showed 5 singlet signals at $\delta$ 7.87,$\delta$ 6.50,$\delta$ 7.33,$\delta$ 6.73 and $\delta$ 6.93 indicating the hydrogen atoms at carbons C-2 ($\delta$ 156.24), C-5 ($\delta$ 102.72), C-8 ($\delta$ 101.97), C-3 ($\delta$ 98.76) and C-6′($\delta$ 116.90). The gHMBC spectrum had cross peaks between 4 methoxy proton groups which had signals at C-2′ $\delta$ 3.75(s), C-4′$\delta$ 3.95(s), C-5′ $\delta$ 3.90(s) and C-6′ ($\delta$ 3.80) and carbons at C-2′ ($\delta$ 153.30), C-4′($\delta$ 152.06), C-5′ ($\delta$ 143.10) and C-6 ($\delta$ 152.92). The DEPT experiment indicated 7 methyl carbon peaks and 3 methylene carbon peaks, which were in the sugar's chemical shifts region of compound **2**.

The aglycone structure of compound **2a**, 7-O-glycosyl-7-hydroxy-2′,4′,5′,6-tetramethoxyisoflavone was similar to that of dalpaniculin-C-glycosylisoflavone from *D. paniculata* seeds, which is 8-C-β-D-glucopyranosyl-5-7-dihydroxy-2′,4′,5′,6-tetramethoxyisoflavone (Rajasekhara Rao and Srinivasa Rao, 1991). However, compound **2a** (aglycone) is linked with the glycone through the oxygen at C-7 in compound **2**, rather than through a C–C bond at C-8.

In order to verify the sugars, the glycoside was acid hydrolyzed, trimethylsilylated and submitted to GC. The gas chromatogram of the trimethylsilyl derivatives of sugars in the sample showed 4 peaks with the same relative retention time as the apiose standard and 2 with the same retention times as those in the glucose standard. The mass fragmentation patterns of these peaks also matched those of the standards and those for glucose matched the α- and β-D-glucopyranose forms in the GC-MS library.

### 2.4. Kinetics and mechanism of hydrolysis of natural substrates

The $K_m$ and $k_{cat}$ values were determined by using *p*NP-β-D-glucoside, *p*NP-β-D-fucoside and the 2 purified natural substrates from *D. nigrescens* seeds (Table 5). The $k_{cat}$ values of native enzyme were calculated per subunit assuming a molecular weight of 62 kDa. The best substrates for the enzyme were the 2 natural *D. nigrescens* glycosides, compounds **1** and **2**. The $K_m$ values were 0.5 mM for compound **1** and 0.7 mM for compound **2**. These $K_m$ values were about 3 and 30 times lower than *p*NP-β-D-fucoside and *p*NP-β-D-glucoside, respectively. In addition, the enzyme showed much higher $k_{cat}$ values for compounds **1** and **2** than for *p*NP-β-D-glucoside and *p*NP-β-D-fucoside. In contrast, *D. cochinchinensis* β-glucosidase, cassava linamarase and almond β-glucosidase could not significantly hydrolyze these substrates (data not shown). The high specificity of *D. nigresecens* β-glycosidase for compounds **1** and **2** and the inability of related enzymes to hydrolyze them indicate that they are likely to be the natural substrates of this enzyme.

Table 5
Kinetic properties of purified *D. nigrescens* β-glycosidase

| Substrate | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| *p*NP-β-D-glucoside | 14.7 | 10.4 | 876 |
| *p*NP-β-D-fucoside | 1.8 | 7.0 | 4020 |
| Compound **1** glycoside | 0.5 | 465 | $9.9 \times 10^5$ |
| Compound **2** glycoside | 0.7 | 334 | $4.4 \times 10^5$ |
| Daidzin | 0.11 | 480 | $4.4 \times 10^6$ |
| Genistin | 0.09 | 500 | $5.6 \times 10^6$ |

The values of $k_{cat}$ and $k_{cat}/K_m$ were estimated assuming a subunit molecular weight of 62 kDa.

When the products of the hydrolysis of compounds **1** and **2** were characterized by TLC, only one sugar spot was seen. This sugar migrated in a position similar to standard disaccharides when run on TLC or when run on GC after trimethylsilylation. When the sugar was purified and hydrolyzed with acid, apiose and glucose were obtained. This indicates the enzyme removed the disaccharide from the glycoside, in contrast to the expectation that it would cut off one sugar at a time, as do most β-glucosidases. Hösel and Barz (1975) previously observed cleavage of biochanin-glucoapioside to biochanin and disaccharide by garbonzo bean isoflavonoid β-glucosidases. It is not so surprising that a family 1 enzyme should be a disaccharidase, since the primeverosidase (EC 3.2.1.149) from tea also belongs to this family (Mizutani et al., 2002). However, since the active site of these enzymes is a funnel-shaped pocket with the sugar binding site on the inside (Barrett et al., 1995), such disaccharidases presumably have a larger pocket for the sugar than monosaccharide-specific enzymes in this family.

In the case of primeverosidase, the enzyme is specific for the disaccharide (Mizutani et al., 2002), while garbonzo bean isoflavonoid β-glucosidase hydrolyzed isoflavonoid glucosides better than apiosoglucosides (Hösel and Barz, 1975). To see if *D. nigrescens* isoflavonoid β-glycosidase preferred glucosides, two isoflavonoid glucosides, daidzin and genistin, were tested. The two were hydrolyzed with $K_m$ values somewhat lower and $k_{cat}$ values somewhat higher than the natural substrates (Table 5), indicating *D. nigrescens* β-glycosidase may prefer glucosides, as well. It is possible such glucoside substrates are also present in the plant, though they were not identified in this study. TLC analysis indicated the enzyme could also hydrolyze 6″-O-malonyl-genticin, though less efficiently than the isoflavonoid glucosides (data not shown).

Thus, the *D. nigrescens* β-glycosidase can hydrolyze isoflavonoid 7-O-β-glucosides, 7-O-β-apiosylglucosides and 7-O-β-malonylglucosides.

## 3. Conclusion

The β-glycosidase purified from *D. nigrescens* Kurz was found to be a glycosyl hydrolase family 1 isoflavonoid

β-apioglucosidase. This is the first time this activity has been described for this family of enzymes, though it includes a wide variety of β-glycosidases. The enzyme also hydrolyzed various *p*NP-glycosides, although with lower activity. The enzyme had a subunit molecular weight of 62–63 kD and appeared to be a tetramer, based on its native molecular weight. These properties, as well as the sequences of peptides from the protein, were similar to *D. cochinchinensis* β-glucosidase. Two substrates of *D. nigrescens* Kurz β-glycosidase were isolated and found to be novel isoflavonoid apiosyl glucosides, from which it removes two sugars at a time, though it can also remove a single glucose from isoflavonoid 7-O-glucosides. This is in contrast to the apparently similar *D. cochinchinensis* Pierre β-glucosidase, for which the substrate is a simple rotenoid glucoside, dalcochinin 12′-O-β-D-glucoside (Svasti et al., 1999). Both enzymes are apparently involved in isoflavonoid metabolism, but the roles of these enzymes from related species may be somewhat different.

## 4. Experimental

### 4.1. General

The structure of the purified compounds were determined by $^{1}$H NMR, $^{13}$C NMR, COSY, gHMBC, gHMQC and DEPT on a 300 MHz Varian UNITY Inova spectrometer (Innova, Darmstadt, Germany). Mass spectrometry of glycosides and aglycones was done on an Esquire-LC Mass spectrometer (Bruker Daltonics, Germany). Thin-layer chromatography and preparative thin-layer chromatography were performed on silica gel 60 $F_{254}$ (Merck, Darmstadt, Germany). Column chromatography was performed with Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). GC–MS analysis of sugars was done on a Varian-CP-3800 Gas Chromatograph and MS-1200L Quadrupole Spectrometer (Varian, CA, USA.) with a Varian-FactorFour Capillary Column, VF-5 ms 30 M × 0.25 MM I.D.

### 4.2. Plant material

*D. nigrescens* seeds were collected from Suranaree University of Technology, Nakhon Ratchasima, Thailand, in January 2003.

### 4.3. Other materials

D(+)-Glucose was purchased from Scharlau (Scharlau Chemie S.A., Barcelona, Spain) and apiose from Omicron (Biochemicals Inc., South Bend, IN, USA). Commercial substrates, 2-mercaptoethanol, polyvinylpolypyrolidine (PVPP), phenyl methyl sulfonyl fluoride (PMSF), and Dowex 2 × 8 resin were obtained from Sigma Chemical (St. Louis, MO, USA). Daidzin, daidzein, genistin, genistein and 6″-O-malonylgenistin were purchased from LC Laboratories (Woburn, MA, USA). All other reagents were analytical grade or better.

### 4.4. Enzyme purification

Seeds were surface sterilized with 0.1% hypochlorite for 10 min, then washed with distilled water and soaked overnight. After this, all procedures were done at 4 °C. Seeds were homogenized with 2 volumes of buffer 1 (0.025 M Tris–HCl, pH 8.0, 70% $(NH_4)_2SO_4$, 10 mM ascorbic acid, 0.025 M β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)). After filtration with cheese cloth, the solids were extracted 2 more times. Then, the solids were extracted with 4 volumes of buffer 2 (0.025 M Tris–HCl, pH 8.0, 0.2 M NaCl, 10 mM ascorbic acid, 0.025 M β-mercaptoethanol, 1 mM PMSF), and centrifuged at 12,000 rpm for 15 min at 4 °C. Polyvinylpolypyrrolidone (PVPP) was added to the supernatant up to 2% and stirred at 4 °C for 1 h. The homogenate was centrifuged at 15,000 rpm for 20 min at 4 °C, and the pellet was discarded. Activated Dowex 2 × 8 resin was added to the supernatant to 25% (w/v), mixed by stirring and removed by centrifugation at 15,000 rpm for 20 min at 4 °C. The protein was fractionated from the supernatant by 35–75% $(NH_4)_2SO_4$ precipitation, and the resulting protein pellet was resuspended in 25 mM Tris–HCl, pH 7.0 before dialysis against the same buffer overnight. The $(NH_4)_2SO_4$ fractionated protein was separated on a DEAE-Sepharose anion exchange column (1.25 × 23.5 cm,115 $cm^3$) with a linear gradient of 0–1 M NaCl in 25 mM Tris–HCl, pH 7.0, at a flow rate of 0.65 ml/min. Fractions containing β-glucosidase and β-fucosidase activity were pooled, concentrated by ultrafiltration (Amicon YM-50, Millipore Corp., Billerica, MA, USA) and fractionated by chromatography in a Sephacryl S-300 (Amersham Pharmacia) gel filtration column (1.5 × 25 cm,150 $cm^3$) equilibrated with 50 mM Tris–HCl pH 7.0, 0.3 M NaCl at a flow rate 0.8 ml/min.

### 4.5. Protein analysis

Protein concentration was determined by the Lowry method (1951) with bovine serum albumin as standard, while column effluents were screened for protein by measurement of $A_{280}$. Denaturing SDS–PAGE was performed by the general method of Laemmli (1970) on 10% polyacrylamide separating gels with Bio-RAD Low-range protein markers (Bio-RAD, Corp., Hercules, CA, USA), and stained with Coomassie Brilliant Blue R250. Non-denaturing polyacrylamide gel electrophoresis, was performed with 7% polyacrylamide separating gels in Laemmli buffer without SDS. Separate gels were stained for β-glucosidase, β-fucosidase and β-galactosi-

dase activities with 1 mM 4-methylumbelliferyl-β-glycosides. The fluorogenic product bands were detected with a Fluor-S$^{TM}$ MultiImager (Bio-RAD). The native molecular weight of the protein was estimated by using Sephacryl S-300 gel filtration chromatography calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) as standards.

The N-terminus of the purified protein was determined on an ABI 473A Peptide Sequencer (Applied Bio-Systems Inc., Foster City, CA, USA). The protein was digested with trypsin (Promega Corp., Madison, WI, USA), and the whole protein or peptides were isolated by HPLC on a Vydac-C4 (4.6 × 250 mm (5 μm)) reverse phase column (Grace Vydac, Columbia, MD, USA), dried onto a glass fiber filter and sequenced.

### 4.6. Determination of β-glycosidase activity

Enzyme reactions were performed in 0.1 M NaOAc buffer, pH 5.0 containing *p*-nitrophenyl-β-D-glycoside. The reaction was incubated at 30 °C for 10 min in the standard assay or various time points for kinetic determinations and stopped with 2 M sodium carbonate. The released *p*-nitrophenol (*p*NP) was determined by measuring absorbance at 400 nm (Montreuil et al., 1986). The pH optimum was determined over the range of pH 3–8.5 in 0.5 pH unit steps in 0.1 M Na citrate, NaOAc, and potassium phosphate buffers at 30 °C. The temperature optimum was determined with 1 mM *p*NP-β-D-glucoside and 1 mM *p*NP-β-D-fucoside in 0.1 M NaOAc over a range of 35–85 °C in a 10 min assay. Hydrolysis of commercially available natural glucosides was measured by glucose release, which was quantitated by a PGO glucose oxidase assay (Sigma Fine Chemicals, St Louis, MO, USA). In the case of daidzin and genistin, the substrates had to be dissolved in DMSO, which was added to 5% or less to the assays. At this concentration, it had little effect on enzyme activity. The PGO assay had to be finished at 10 min to avoid interference. Enzyme kinetics were analyzed by linear regression using the method of Lineweaver and Burk (1934) and by non-linear regression with the Enzfitter computer program (Elsevier Biosoft, Cambridge, UK).

### 4.7. Effects of metal ions and inhibitors on enzyme activity

The effects of some metal ions and inhibitors on activity were determined by pre-incubating the purified enzyme with the substance for 10 min at 30 °C in 0.1 M sodium acetate, pH 5.0, then adding 1 mM of *p*NP-β-D-fucoside or 2 mM *p*NP-β-D-glucoside and incubating for 10 min at 30 °C. For δ-gluconolactone, assays were done without preincubation and the inhibitor was freshly prepared, since it is susceptible to break-down in water (Combes and Birch, 1988).

### 4.8. Compound **1** and **2** extraction and isolation

The powder was extracted with 3 volumes of absolute methanol by stirring overnight at room temperature. The extract was filtered and partitioned with 1 volume of hexane. The methanol fraction was dried by rotary evaporator and the residue dissolved with water. The water fraction was extracted with 2.5 volumes of ethyl acetate and then dried by speed vacuum. The dried residue was dissolved with methanol and separated by chromatography on a 1.2 × 85 cm Sephadex LH-20 gel filtration column. The compounds were eluted with methanol collecting 5 mL fractions, and the fractions containing β-glucosidase substrates were pooled. The β-glucosidase substrates were identified by incubating aliquots of fractions (10% by volume) with and without β-glucosidase in 0.1 M NaOAc (pH 5.0) 10 min at 30 °C. The products were separated on analytical silica gel 60 $F_{254}$ aluminum TLC sheets developed twice with solvent A, EtOAc/methanol/$H_2O$/acetic acid 15:2:1:2 (by vol). The sheets were visualized under 254 nm wavelength UV light to identify spots with shifted mobility after enzymatic digest.

Compounds **1** and **2** were purified using the preparative thin-layer chromatography by developing twice with solvent A. The LH-20 column fractions containing compound **1** were incubated overnight in 0.1 M NaOAc buffer, pH 5.0, with *D. cochinchinensis* β-glucosidase to hydrolyze low abundance glycoside contaminants before TLC purification of the compound **1** glycoside. The compounds **1** and **2** glycosides were digested with 0.1 unit of *D. nigrescens* β-glucosidase to the aglycones by incubating in 0.1 M sodium acetate buffer pH 5.0 at 30 °C, overnight. The aglycone of compound **2** was purified by preparative silica gel 60 $F_{254}$ TLC using solvent B, EtOAc/methanol/$H_2O$ 5:2:1 (by vol).

### 4.9. HPLC and TLC analysis of hydrolysis products

These natural substrates, S1 and S2 were subjected to enzyme hydrolysis in the standard assay described above. The reaction products were separated and quantified with an Eclipse XDB-C18 (4.6 × 250 mm, 5 μm) reverse phase column on an HP-Series 1100 HPLC (Agilent Corp, Palo Alto, CA, USA) with a linear gradient of 0–100% methanol in 0.1% TFA/water.

TLC of hydrolyzed products was performed on analytical silica gel 60 $F_{254}$ aluminum plates (Merck, Darmstadt, Germany) with $CHCl_3$/MeOH/$H_2O$ (15:3:1) as solvent.

### 4.10. Sugar analysis

Glycoside (5.5 mg) was boiled with 0.7 mL 0.1 N $H_2SO_4$ in a 1.5 mL microcentrifuge tube for 25 min. The solution was cooled and extracted with n-butanol,

then neutralized with $NaHCO_3$ and the aqueous layer was dried by rotary evaporator. The trimethylsilylation of sugar was done by the method of Nikolov and Reilly (1983) with three milligrams of dried residue. The trimethylsilylation mixture was injected into the gas chromatograph and separated with the following conditions: injector at 250 °C; split ratio 20:1; helium carrier gas at 1.2 mL/min; the column temperature was held at 150 °C for 5 min and increased to 190 °C at 20 °C/min and held for 20 min. Mass spectra were collected with a source temperature of 200 °C, a source voltage of 70 eV, and $3.2 \times 10^{-7}$ Torr.

### 4.11. Compound **1**, dalpatein-7-O-[β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside]

Compound **1** was obtained as an amorphous light yellow powder: m.p. 158–165 °C; UV (MeOH) $\lambda_{max}$ nm: 260 and 315; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3435 (OH), 1709, 1639, 1559, 1412, 1270, 795, 639; For $^{1}H$ and $^{13}C$ NMR spectra, see Table 1. FT-MS *m/z* 659 $[M + Na]^+$ calculated for $C_{29}O_{16}H_{32}$.

### 4.12. Compound **2**, dalnigrein-7-O-[β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranoside]

Compound **2** was obtained as an amorphous light yellow powder: m.p. decomposed at 160–200 °C; UV (MeOH) $\lambda_{max}$ nm: 260 and 315; IR (KBr) $\nu_{max}$ $cm^{-1}$: 3436 (OH), 1706, 1639, 1561, 1412, 808, 643, 524; For $^{1}H$ and $^{13}C$ NMR spectra, see Table 1. FT-MS *m/z* 675 $[M + Na]^+$ calculated for $C_{30}O_{16}H_{36}$.

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