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Purification and Characterization of Vanilla Bean (*Vanilla planifolia* Andrews) β-D-Glucosidase

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Vanilla bean β-D-glucosidase was purified to apparent homogeneity by successive anion exchange, hydrophobic interaction, and size-exclusion chromatography. The enzyme is a tetramer (201 kDa) made up of four identical subunits (50 kDa). The optimum pH was 6.5, and the optimum temperature was 40 °C at pH 7.0. K_m values for *p*-nitrophenyl-β-D-glucopyranoside and glucovanillin were 1.1 and 20.0 mM, respectively; V_{max} values were 4.5 and 5.0 μkat·mg^{-1}. The β-D-glucosidase was competitively inhibited by glucono-δ-lactone and 1-deoxynojirimycin, with respective K_i values of 670 and 152 μM, and not inhibited by 2 M glucose. The β-D-glucosidase was not inhibited by *N*-ethylmaleimide and DTNB and fully inhibited by 1.5−2 M 2-mercaptoethanol and 1,4-dithiothreitol. The enzyme showed decreasing activity on *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-β-D-xylopyranoside. The enzyme was also active on prunasin, esculin, and salicin and inactive on cellobiose, gentiobiose, amygdalin, phloridzin, indoxyl-β-D-glucopyranoside, and quercetin-3-β-D-glucopyranoside.

KEYWORDS: ***Vanilla planifolia* Andrews; vanilla bean; β-glucosidase; glucovanillin hydrolysis**

INTRODUCTION

Upon natural ripening of vanilla beans, whether on the vine or during curing (*1*−*3*), a β-glucosidase is responsible for the hydrolysis of nonvolatile glycosidic aroma precursors consisting mainly of vanillin β-glucoside (or glucovanillin) and minor glycosides of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid (*4*), vanillic acid, and *p*-hydroxybenzyl alcohol (*5*). The aglycons, mostly vanillin, thereby released are responsible for the strong vanilla flavor of the beans. The traditional method of curing vanilla beans in the Democratic Republic of Madagascar, Réunion Island, and Indonesia starts by a killing step, whereby mature vanilla beans are put in a hot water bath (60 °C for 3 min), immediately followed by a sweating period during which beans are placed in cloth-lined wooden boxes for 24 h at ambient temperature. The killing step is performed to prevent subsequent dehiscence of the beans during drying. After this sequence has been repeated, vanilla beans are dried for 3 months by exposing them to the morning sun and storing them in the shade during the afternoon (*3*). However, during the initial killing−sweating steps of the curing process, only 40% of the glucovanillin is hydrolyzed into vanillin (*3*). Furthermore, during the drying period, the vanillin content (as a proportion of dry matter) no longer increases from that reached after the initial steps.

There is little data concerning vanilla bean β-glucosidase, with only a few papers mentioning the level of activity upon ripening (*1*, *2*, *6*), during the curing process (*1*, *2*, *7*) or in extracts obtained under various conditions (*8*); to our knowledge, the β-glucosidase activity has not yet been purified. The objective of this work was to purify vanilla bean β-glucosidase and characterize some of its properties to better understand the events occurring during the curing process.

MATERIALS AND METHODS

Plant Material. Several batches of mature green vanilla beans were harvested in the Comores Islands between June and August 2001, immediately air-freighted to our laboratory, and treated upon arrival.

Chemicals. *p*-Nitrophenyl glycosides, 2-mercaptoethanol, 1,4-dithiothreitol, *N*-ethymaleimide, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 1-deoxynojirimycin, glucono-δ-lactone, salicin, amygdalin, esculin, phloridzin, cellobiose, gentiobiose, indoxyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, Fast Blue BB, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and molecular weight markers were from Sigma (St. Louis). Prunasin and quercetin-3-β-D-glucopyranoside were from Extrasynthèse (Genay, France). Octyl-β-D-glucopyranoside was from Fluka (Basel, Switzerland). DEAE Sepharose Fast Flow, Phenyl Sepharose Fast Flow, and HiPrep Sephacryl S-300 HR were from Amersham Biosciences Europe GmbH (Saclay, France). All other reagents were of the best available grade.

Purification of Glucovanillin. Vanilla beans (160 g) were ground with a Waring blendor in 96% ethanol (500 mL). The slurry was boiled for 30 min, and the medium was filtered on Whatman filter paper and brought to dryness in a vacuum rotary evaporator. The residue was dissolved in distilled water and extracted with methylene chloride (4 × 100 mL). The aqueous phase was injected onto an XAD-2 column (5 × 20 cm) (Rohm and Haas France S.A., Chauny, France); after the column was washed with water (500 mL), ethanol (250 mL) was passed, and the medium was brought to dryness. The residue was then dissolved

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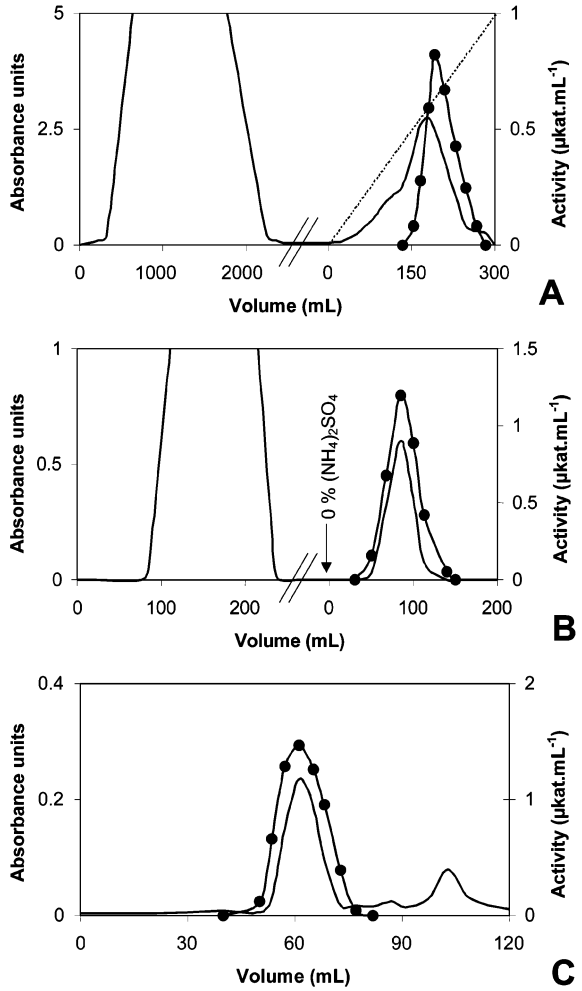

Figure 1. (A) DEAE Sepharose Fast Flow chromatography, (B) Phenyl Sepharose Fast Flow chromatography of pooled, active fractions from DEAE Sepharose Fast Flow chromatography, and (C) HiPrep Sephacryl S-300 HR size-exclusion chromatography of pooled, concentrated active fractions from Phenyl Sepharose Fast Flow chromatography [(—) protein at 280 nm, (●) β-glucosidase activity, (•••) 0 → 0.5 M NaCl gradient].

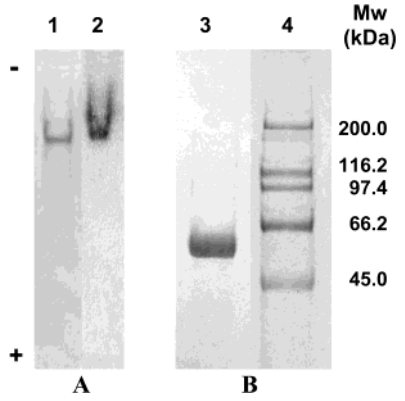

Figure 2. (A) Native PAGE of the vanilla bean β-glucosidase: lane 1, protein staining with Coomassie Brilliant Blue G-250 (10 μg of protein); lane 2, activity staining with 6-bromo-2-naphthyl-β-D-glucopyranoside and Fast Blue BB (15 μg of protein). (B) SDS–PAGE of the vanilla bean β-glucosidase, protein staining with Coomassie Brilliant Blue G-250: lane 3, β-glucosidase (5 μg of protein); lane 4, molecular weight markers.

in methanol (30 mL), and the solution was kept at −18 °C for 48 h. After crystallization of glucovanillin, the methanolic supernatant was discarded. Crystals were then washed on a fritted crucible with ethyl acetate and redissolved in distilled water (50 mL), and the solution

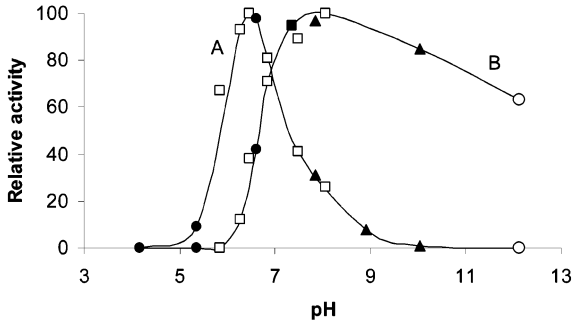

Figure 3. (A) β-Glucosidase activity as a function of pH [(●) 0.2 M citric acid–NaOH; (□) 0.066 M KH_2PO_4–Na_2HPO_4; (■) 0.05 M Tris–HCl; (▲) 0.05 M KCl–boric acid–NaOH; (○) 0.05 M KCl–NaOH buffers; 40 °C, 20 min, 0.05 nkat]. (B) β-Glucosidase stability as a function of pH [incubation of 1 nkat in the above buffers for 4 h at 20 °C and then measurement of activity under standard conditions (0.1 M phosphate buffer, pH 7.0, 40 °C, 20 min)].

was passed through a Supelclean LC-18 SPE column (6 mL) (Supelco, Pennsylvania). The solution was then brought to dryness; glucovanillin crystals were then dried overnight in a vacuum oven (50 °C). Yield: 240 mg. The purity was estimated after total hydrolysis with sweet almond β-glucosidase by measuring the vanillin content by HPLC (*3*): 96%.

Enzyme Assay. β-Glucosidase activity was determined by incubating 0.2 mL of 4 mM *p*-nitrophenyl-β-glucopyranoside (pNPG) in 0.1 M sodium phosphate buffer (pH 7.0) with 0.2 mL of enzyme diluted in the same buffer (<0.2 nkat in the reaction medium) for 20 min at 40 °C. The reaction was stopped by the addition of 1.0 mL of 0.5 M NaOH, and the absorbance was read at 400 nm. One nkat of β-glucosidase activity is the amount of enzyme that hydrolyzes 1 nmol of substrate/s at pH 7.0 (40 °C).

Protein Determination. Proteins were recorded in column effluents at 280 nm or measured by the Lowry method (*9*) using bovine serum albumin as a standard after precipitation with trichloroacetic acid (7.5% final concentration), centrifugation (1000*g*, 5 min), and subsequent dissolution of the pellet in 0.5 M NaOH (10 min, boiling water).

Purification of the β-Glucosidase. The whole purification procedure was conducted at 20 °C.

(a) Extraction. Vanilla beans (150 g) were homogenized in a Waring blendor with 1.5 L of 20 mM Tris−HCl buffer (pH 8.0), and the slurry was filtered on muslin.

(b) Anion Exchange Chromatography. The glucosidase extract (1.1 L) was loaded at 10 mL·min^{-1} onto a DEAE Sepharose Fast Flow column (2.6 × 20 cm) equilibrated with 20 mM Tris−HCl buffer (pH 8.0), and the column was washed with the same buffer until zero UV absorbance. A linear gradient (0 → 0.5 M) of NaCl (300 mL) in the same Tris−HCl buffer was then applied. Fractions (10 mL) were collected (**Figure 1A**).

(c) Hydrophobic Interaction Chromatography. Active fractions from (b) eluted in the range 0.25−0.45 M NaCl were combined (150 mL), $(NH_4)_2SO_4$ was added to 1 M final concentration, and the mixture was injected at 5 mL·min^{-1} on a Phenyl Sepharose Fast Flow column (2.6 × 12 cm) previously equilibrated with 20 mM Tris−HCl buffer (pH 8.0) containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer until zero UV absorbance and then eluted with 20 mM Tris−HCl buffer (pH 8.0). Fractions (3 mL) were collected (**Figure 1B**).

(d) Size-Exclusion Chromatography. Active fractions from (c) were combined (48 mL) and concentrated to 4 mL with a Macrosep 3K Omega centrifugal device (molecular weight cutoff 3000; Pall, Michigan) for 90 min at 5000*g*, NaCl was added to 0.15 M final concentration, and the mixture was injected at 30 mL·h^{-1} onto a HiPrep Sephacryl S-300 HR column (1.6 × 60 cm) previously equilibrated with 20 mM Tris−HCl buffer (pH 8.0) containing 0.15 M NaCl. Fractions (2 mL) were collected (**Figure 1C**).

Electrophoresis. PAGE (10 μg for protein staining and 15 μg for activity staining) was conducted in a Mini-Protean II dual-slab electrophoresis cell (Bio-Rad, California) on 7.5% acrylamide gels.

Table 1. Purification of Vanilla Bean β-D-Glucosidase

step	vol (mL)	activity (nkat·mL^{-1})	protein concn (mg·mL^{-1})	total activity (μkat)	total protein amt (mg)	specific activity (μkat·mg^{-1})	activity yield (%)	purification factor
crude extract	1100	151	0.37	166.1	407.0	0.41	100.0	1.0
DEAE Sepharose	150	423	0.23	63.5	34.5	1.84	38.2	4.5
Phenyl Sepharose	48	672	0.28	32.3	13.4	2.41	19.4	5.9
Sephacryl S-300 HR	16	869	0.29	13.9	4.7	2.96	8.4	7.2

SDS−PAGE (5 μg of purified β-glucosidase) was performed according to Laemmli (*10*) on 12% acrylamide gels. Molecular weight markers were ovalbumin (M_w 45000), bovine serum albumin (66200), phosphorylase B (97400), β-galactosidase (116200), and myosin (200000). The gels were stained with Coomassie Brilliant Blue G-250 according to the manufacturer directions (Bio-Rad). β-Glucosidase activity was revealed in native PAGE gels with 6-bromo-2-naphthyl-β-D-glucopyranoside and Fast Blue BB (*11*).

Molecular Weight Determination. The molecular weight of the native enzyme was determined using a HiPrep Sephacryl S-300 HR column (1.6 × 60 cm) eluted at 30 mL·h^{-1} with 20 mM Tris−HCl (pH 8.0) containing 0.15 M NaCl. The column was calibrated with ovalbumin (M_w 45000), phosphorylase B (97400), aldolase (168000), ferritin (440000), and thyroglobulin (669000). The subunit molecular weight was determined by SDS−PAGE.

Effect of pH. The pH optimum was determined by measuring β-glucosidase activity under standard conditions (0.05 nkat in the assay) using 0.2 M citric acid−NaOH, 0.1 M KH_2PO_4−Na_2HPO_4, 0.05 M Tris−HCl, 0.05 M KCl−boric acid−NaOH, and 0.05 M KCl−NaOH buffers. pH stability was measured under standard conditions after incubation of the purified β-glucosidase (1 nkat) for 4 h at 20 °C in the above buffers.

Effect of Temperature. The temperature optimum was determined by measuring β-glucosidase activity under standard conditions (0.05 nkat) in the temperature range 20−70 °C. Temperature stability was measured under standard conditions after incubation of the purified β-glucosidase (1 nkat) in 0.1 M phosphate buffer (pH 7.0) for 30 min at different temperatures (20−70 °C).

Kinetic Parameters. The activity of the purified β-glucosidase was measured under standard conditions (0.05 nkat) with the final concentration of *p*-nitrophenyl-β-glucopyranoside or glucovanillin varying from 0.5 to 60 mM and from 2.5 to 50 mM, respectively. Measurements were carried out in triplicate.

Effect of Various Reagents. The purified β-glucosidase (0.05 nkat in 200 μL of 0.1 M sodium phosphate buffer, pH 7.0) was incubated for 60 min at 4 °C with various concentrations of the following reagents: 10 mM 5,5′-dithiobis(2-nitrobenzoic acid), 25 mM *N*-ethylmaleimide, and 10 mM EDTA. The residual activity was then measured under standard conditions; since DTNB developed interfering coloration in the standard assay, 4 mM glucovanillin was used as substrate in place of *p*NPG. Released vanillin was measured by HPLC (*3*). Reagents in the 400 μL assay volume were therefore at half their initial concentration. The purified β-glucosidase (1 nkat) was also incubated for 60 min at 4 °C in the presence of 0.05−2 M 2-mercaptoethanol or 1,4-dithiothreitol in 0.1 M sodium phosphate buffer (pH 7.0); residual activity was then measured under standard conditions (*p*NPG as substrate).

Effect of Metal Ions. The purified β-glucosidase (0.05 nkat in 200 μL of 50 mM HEPES−NaOH buffer, pH 7.0) was preincubated for 30 min at 4 °C in the presence of various concentrations of metal ions. The activity was then measured under standard conditions but using 50 mM HEPES−NaOH buffer (pH 7.0) in place of 0.1 M phosphate buffer due to the poor solubility of most metal ions in phosphate buffer. Ions in the 400 μL assay volume were therefore at half their initial concentration.

Substrate Specificity. The activity of the purified β-glucosidase was measured under standard conditions (0.1 nkat) with various substrates at 2 mM final concentration. For some substrates, glucose released by the enzyme was determined by the glucose oxidase/peroxidase/*o*-dianisidine system (*12*). Aglycons released from some substrates by the enzyme were measured by HPLC.

RESULTS AND DISCUSSION

It should first be mentioned that the purification procedure gave very similar results when applied to other batches of mature green vanilla beans originating from Mexico and the Democratic Republic of Madagascar. Furthermore, extraction was conducted on fresh beans since we found in a preliminary experiment that freezing strongly lowers the β-glucosidase activity. The extraction of β-glucosidase from vanilla beans was performed in 20 mM Tris−HCl buffer (pH 8.0) without the addition of protective agents such as polyvinylpolypyrrolidone, 2-mercaptoethanol, ascorbic acid, or protease inhibitors, as levels of activity were stable and similar in extracts obtained with and without protective agents. The extraction was performed at pH 8.0 since preliminary experiments had shown that the activity was very unstable in acidic conditions. Contrary to previously reported extraction conditions (v/w = 1; ref *8*), we used a buffer volume to mass of vanilla beans ratio equal to 10 since we showed in a preliminary experiment that the β-glucosidase was very unstable at lower ratios. Since the β-D-glucosidase activity in crude extracts was much more stable at ambient temperature than in the cold, the whole purification procedure was conducted at 20 °C.

Most proteins present in the extract did not bind to DEAE Sepharose Fast Flow (**Figure 1A**), while the β-glucosidase was eluted between 0.25 and 0.4 M NaCl. Active fractions were pooled, $(NH_4)_2SO_4$ was added to 1 M final concentration, and the mixture was injected onto a Phenyl Sepharose column. Once again most proteins did not bind to the hydrophobic exchanger, while the enzyme was strongly adsorbed. Since preliminary experiments had shown that no proteins were eluted over a decreasing $(NH_4)_2SO_4$ linear gradient (1 M → 0), the activity was eluted by passing 20 mM Tris−HCl buffer (pH 8.0) (**Figure 1B**). Active fractions were pooled, concentrated with a Macrosep 3K Omega centrifugal device, and injected onto a Sephacryl S-300 HR column. Some inactive proteinaceous material was observed, while the β-glucosidase activity was eluted at around 60 mL (**Figure 1C**). A summary of the purification of vanilla bean β-glucosidase is presented in **Table 1**.

At this stage of purification, the β-glucosidase appeared as electrophoretically homogeneous, giving a single protein band (Coomassie Brilliant Blue staining) in native PAGE (**Figure 2A**). On the same gel, after staining for β-glucosidase activity using 6-bromo-2-naphthyl-β-D-glucopyranoside and Fast Blue BB, a single red, diffuse band was observed at the same migration distance (Figure 2A). When electrophoresed in SDS−PAGE, the β-glucosidase gave a single band at 50 kDa molecular mass (**Figure 2B** and **Table 2**). The molecular mass of the native β-glucosidase was also determined by size-exclusion chromatography on a calibrated Sephacryl S-300 HR column and found to equal 201 kDa (**Table 2**), indicating that

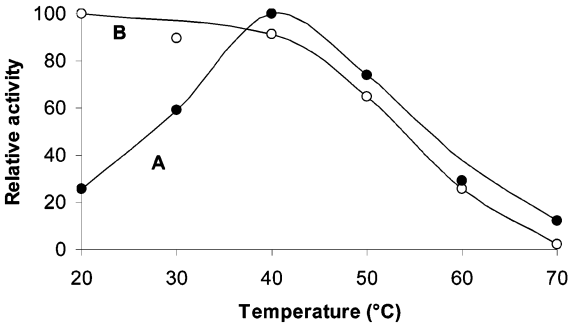

Figure 4. (A) β-Glucosidase activity as a function of temperature (0.1 M phosphate buffer, pH 7.0, temperature range of 20–70 °C, 20 min). (B) β-Glucosidase stability as a function of temperature [incubation of 1 nkat in 0.1 M phosphate buffer (pH 7.0) for 30 min at different temperatures (20–70 °C) and then measurement of activity under standard conditions (0.1 M phosphate buffer, pH 7.0, 40 °C, 20 min)].

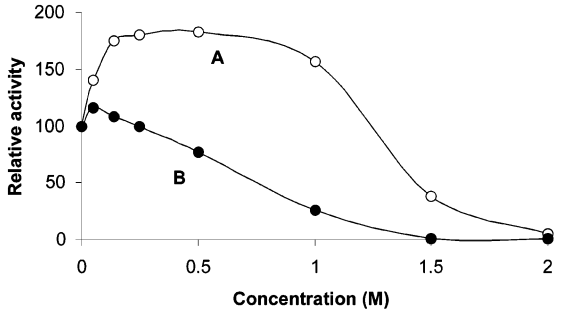

Figure 5. Effect of (A) 2-mercaptoethanol and (B) 1,4-dithiothreitol on enzyme activity. The β-glucosidase (1 nkat) was exposed for 1 h at 4 °C to different concentrations of the reducing agents in a total volume of 200 μL of 0.1 M phosphate buffer, pH 7.0. Then the residual activity was measured under standard conditions (0.1 M phosphate buffer, pH 7.0, 40 °C, 20 min) and expressed as a percent of activity measured without reducing agent.

Table 2. Properties of Vanilla Bean β-D-Glucosidase

property	
molecular mass (kDa)[a]	201
subunit molecular mass (kDa)[b]	50
optimum pH	6.5
optimum Θ (°C)	40
glycosylation[c]	+
p-Nitrophenyl-β-D-glucopyranoside	
V_{max} (40 °C, pH 7) (μkat·mg^{-1})	4.5
K_m (40 °C, pH 7) (mM)	1.1 ± 0.2
Glucovanillin	
V_{max} (40 °C, pH 7) (μkat·mg^{-1})	5.0
K_m (40 °C, pH 7) (mM)	20.0 ± 2.0
Glucono-δ-lactone	
K_i (40 °C, pH 7, *p*NPG as substrate) (μM)	670, competitive inhibition
1-Deoxynojirimycin	
K_i (40 °C, pH 7, *p*NPG as substrate) (μM)	152, competitive inhibition

[a] Determined by size exclusion chromatography on Sephacryl S-300 HR. [b] Determined by SDS–PAGE. [c] Tested by the phenol-sulfuric acid method (*18*).

the vanilla bean β-glucosidase is a tetramer built of four identical subunits. The subunit molecular mass of vanilla bean β-glucosidase is similar to the molecular masses of monomeric glucosidases (*13*, *14*) or of subunits of polymeric glucosidases (*15*−*17*). Glycosylation of the vanilla β-glucosidase was tested positive by the phenol−sulfuric acid method (*18*) (**Table 2**).

The vanilla bean β-glucosidase exhibited a sharp optimum pH curve with a maximum at pH 6.5 (**Figure 3**), a result in agreement with the previously determined optimum pH on crude extracts of vanilla beans from Costa Rica (*2*) and Reunion Island (*19*). An optimal pH of 4.0, however, was reported for crude enzyme extracts from Indonesian vanilla beans (*7*). The vanilla bean β-glucosidase was shown to be very unstable below neutrality since it lost its activity below pH 6.0 after 4 h at 20 °C (**Figure 3**); conversely, it was found very stable under neutral and alkaline pH since it retained up to 63% of its activity at pH 12 under the same conditions. The vanilla bean β-glucosidase exhibited an optimum temperature of 40 °C and is fully inactivated after 30 min at 70 °C in 0.1 M phosphate buffer (pH 7.0) (**Figure 4**). The purified enzyme retained its activity for at least 6 months in 20 mM Tris−HCl buffer (pH 8.0) at 4 °C.

The K_m for *p*-nitrophenyl-β-D-glucopyranoside was 1.1 mM (0.1 M phosphate buffer, pH 7.0; 40 °C) (**Table 2**), a value similar to those reported for rubber tree leaf (*20*) and butter bean (*21*) β-glucosidases and differing from a previously reported K_m (0.38 mM) measured using crude enzyme extracts of Indonesian vanilla beans (0.1 M acetate buffer, pH 4.0; 30 °C) (*7*). The V_{max} was high (4.5 μkat·mg^{-1}) and comparable to that obtained for butter bean glucosidase (*21*). The K_m and V_{max} for glucovanillin (natural substrate) were 20.0 mM and 5.0 μkat·mg^{-1}, respectively. The vanilla bean β-glucosidase was competitively inhibited by glucono-δ-lactone and 1-deoxynojirimycin with apparent K_i values of 670 and 152 μM, respectively (**Table 2**). Thus, this β-glucosidase is only weakly inhibited by 1-deoxynojirimycin compared to sweet almond β-glucosidase (pH-corrected $K_i = 6.5$ μM) (*22*). Interestingly, the vanilla bean β-glucosidase was not inhibited by glucose up to 2 M.

When residual activity was measured after vanilla bean β-glucosidase had been incubated for 60 min at 4 °C in the presence of 0.05−2 M 2-mercaptoethanol, it was found to be enhanced up to 2× at 0.5 M (**Figure 5**), yet 95% of β-glucosidase activity was lost at 2 M. Very similar behavior (i.e., activation up to 1.3× at 0.5 M and then loss of activity at 2 M) was observed for the maize coleoptile β-glucosidase when preincubated with 2-mercaptoethanol (*23*). A small enhancement of activity (1.2×) was also observed when the enzyme was exposed to 50 mM 1,4-dithiothreitol, with almost complete activity loss at 1.5 M. Therefore, although the activation of β-glucosidase at low to moderate concentrations of reducing agents remains poorly understood, the inactivation occurring at high concentrations suggests that the vanilla bean β-glucosidase requires disulfide bonds for activity. Similar to sweet almond β-glucosidase (*24*), the vanilla bean enzyme was strongly inhibited (96%) by Cu^{2+} at 1 mM and also by Zn^{2+} (**Table 3**). Mg^{2+} and Ca^{2+} little affected the enzyme activity, while Co^{2+}, Mn^{2+}, and Ni^{2+} inhibited the activity by 50−70%. Hg^{2+} had a pronounced inhibitory effect since almost all the activity is lost at 60 μM. Contrary to maize coleoptile β-glucosidase, which is fully inhibited by 256 μM Hg^{2+} and 32 μM Ag^{+} (*23*), Ag^{+} is not inhibitory at 0.1 mM and decreased the activity by 70% at 1 mM. Although the enzyme is strongly inhibited by Hg^{2+}, absence of inhibition by thiol reagents, DTNB and *N*-ethymaleimide, suggests that no accessible SH groups are involved in activity. EDTA did not inhibit the activity.

Despite its electrophoretic homogeneity, the vanilla bean β-glucosidase exhibited activity not only on *p*-nitrophenyl-β-D-glucopyranoside but also on *p*-nitrophenyl-β-D-fucopyranoside, β-D-galactopyranoside, and β-D-xylopyranoside (Table 4) with relative activities at 2 mM of 1:2.15:0.61:0.05. Similar ratios were found for these four substrates in the β-glucosidases

Table 3. Effect of Metal Ions on Vanilla Bean β-D-Glucosidase

ion	concn tested (mM)	relative activity[a]	ion	concn tested (mM)	relative activity[a]
Hg^{2+}	0.001	78	Mg^{2+}	1	89
	0.005	58	Ca^{2+}	1	82
	0.02	29	Co^{2+}	1	47
	0.04	6	Mn^{2+}	1	34
	0.06	3	Ni^{2+}	1	30
Ag^{+}	0.1	112	Zn^{2+}	1	7
	1	29	Cu^{2+}	1	4

[a] The β-glucosidase (0.05 nkat in 200 μL of 50 mM HEPES–NaOH buffer, pH 7.0) was exposed for 30 min at 4 °C to different concentrations of metal ions. The activity was then measured under standard conditions and expressed relative to the activity measured on *p*-nitrophenyl-β-D-glucopyranoside (100) without added ion.

Table 4. Relative Activity of Bean β-D-Glucosidase on Various Substrates

substrate	relative activity[a]
p-nitrophenyl-β-D-glucopyranoside	100
p-nitrophenyl-β-D-fucopyranoside	215
p-nitrophenyl-β-D-galactopyranoside	61
p-nitrophenyl-β-D-xylopyranoside	5
p-nitrophenyl-β-D-mannopyranoside	nd[b]
p-nitrophenyl-α-L-arabinofuranoside	nd
p-nitrophenyl-α-L-rhamnopyranoside	nd
p-nitrophenyl-α-D-glucopyranoside	nd
glucovanillin[c]	22
prunasin[c]	300
esculin[c]	92
salicin[c,d]	3.6
phloridzin[c,d]	nd
indoxyl-β-D-glucopyranoside[c,d]	nd
quercetin-3-β-D-glucopyranoside[c–e]	nd
amygdalin[d,f]	nd
octyl-β-D-glucopyranoside[d,f]	0.4
cellobiose[d,f]	nd
gentiobiose[d,f]	nd

[a] 2 mM substrate concentration, 0.1 M sodium phosphate buffer (pH 7.0), 20 min, 40 °C, 0.1 nkat of enzyme. Activity expressed relative to activity measured on *p*-nitrophenyl-β-D-glucopyranoside (100). [b] Not detected. [c] Released aglycon measured by HPLC. [d] Incubated with the enzyme for 5 h. [e] Assayed in the presence of 10% ethanol. [f] Released glucose measured by the glucose oxidase/peroxidase/*o*-dianisidine system.

from butter bean, *Phaseolus lunatus* (*21*), and wheat seedlings (*17*). Broad specificity of plant β-glucosidases with regard to *p*-nitrophenyl glycosides was also observed in sweet cherry (*25*), maize (*26*), and *Polygonum tinctorium* leaves (*27*) with maximum activity found on *p*-nitrophenyl-β-D-fucopyranoside. No activity was found on *p*-nitrophenyl-β-D-mannopyranoside, α-L-arabinofuranoside, α-L-rhamnopyranoside, and α-D-glucopyranoside. Like rubber tree (*20*) and maize (*26*) β-glucosidases, the vanilla bean β-glucosidase does not hydrolyze glycosyl glucosides, cellobiose, gentiobiose, and amygdalin (D-mandelonitrile-β-gentiobioside). Thus, according to its specificity, this vanilla enzyme can be viewed more as a β-D-glycosidase than as a β-D-glucosidase. Rates of hydrolysis of prunasin, esculin, glucovanillin, and salicin at 2 mM were 3.00:0.92:0.22:0.04 relative to *p*NPG, which was unexpected since the aglycon of salicin is structurally closer to vanillin than esculin. Similar specificity was observed in the case of wheat seedling β-glucosidase (*17*). Phloridzin, indoxyl-β-D-glucopyranoside, and quercetin-3-β-D-glucopyranoside were not substrates. Octyl-β-D-glucopyranoside was hydrolyzed at a very slow rate (0.4% vs *p*NPG).

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