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Identification of glucosides in green beans of *Vanilla planifolia* Andrews and kinetics of vanilla β-glucosidase

Mark J.W. Dignum[a], Rob van der Heijden[b], Josef Kerler[c], Chris Winkel[c], Rob Verpoorte[a,*]

[a]*Division of Pharmacognosy, Leiden University, Einsteinweg 55, PO Box 9502, 2300 RA Leiden, The Netherlands*
[b]*Division of Analytical Chemistry, Leiden University, Einsteinweg 55, PO Box 9502, 2300 RA Leiden, The Netherlands*
[c]*Quest International Nederland B.V., Huizerstraatweg 28, 1411 GP Naarden, the Netherlands*

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Abstract

Natural vanilla is extracted from the fruits of *Vanilla planifolia*. In the overall vanilla aroma, minor compounds *p*-cresol, creosol, guaiacol and 2-phenylethanol have a high impact. This is shown by GC-Olfactometry analysis of cured vanilla beans. The presence of β-D-glucosides of these compounds was investigated, in order to determine if these compounds are derived from glucosides or if they are formed during the curing process via different pathways. Glucosides of vanillin, vanillic acid, *p*-hydroxy benzaldehyde, vanillyl alcohol, *p*-cresol, creosol and bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate and bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2-butyl)tartrate have been identified in a green bean extract. The kinetics of the β-glucosidase activity from green vanilla beans towards eight glucosides naturally occurring in vanilla and towards *p*-nitrophenol were investigated. For glucosides of *p*-nitrophenol, vanillin and ferulic acid the enzyme had a K_m of about 5 mM. For other glucosides (vanillic acid, guaiacol and creosol) the K_m-values were higher (>20 mM). The V_{max} was between 5 and 10 IU mg^{-1} protein for all glucosides tested. Glucosides of 2-phenylethanol and *p*-cresol were not hydrolysed. β-Glucosidase does not have a high substrate specificity for the naturally occurring glucosides compared to the synthetic *p*-nitrophenol glucoside (K_m 3.3 mM, V_{max} 11.5 IU mg^{-1} protein).

Keywords: *Vanilla planifolia* Andrews; Orchidaceae; β-glucosidase; Enzyme kinetics; β-D-glucosides; Glucoside synthesis

1. Introduction

The characteristic aroma of vanilla is only obtained after a curing process of green fruits of the orchid *Vanilla planifolia* Andrews. The harvested fruits, called beans or pods, contain many different glucosidic compounds. The curing process is meant to hydrolyse the glucosides and to release the aroma compounds. β-Glucosidases are believed to play an important role in this process (Arana, 1943). The β-glucosidase activity in vanilla beans is highest six or seven months after pollination (Arana, 1943; Wild-Altamirano, 1969) and also the amount of glucosides is at its highest level then (Kanisawa, 1993). On the other hand, β-glucosidase activity is lost during the curing process (Dignum, Kerler, & Verpoorte, in press; Ranadive, Szkutnica, Guerrera, & Frenkel, 1983), indicating that the aroma formation might not be a completely enzymatic process.

In scientific literature, only a few references can be found on the characteristics of β-glucosidase in vanilla. It is not known if the measured activity is caused by only one enzyme, or if there are several β-glucosidases present. Some data on the fate of β-glucosidase activity during curing are available (Dignum et al., in press; Ranadive et al., 1983). Hanum (1997) published data on the activity of a partly purified β-glucosidase towards the synthetic β-glucosidase substrate *p*-nitrophenol glucoside. So far there are no publications on experiments on the kinetics of the β-glucosidase activity towards the different glucosides naturally occurring in vanilla beans.

The aim of this research is to investigate the β-glucosidase and its selectivity for several glucosides that are present in green beans. The chemical synthesis of the glucosides and their identification in vanilla beans is

* Corresponding author. Tel.: +31-71-527-4528; fax: +31-71-527-4511.
E-mail address: verpoort@chem.leidenuniv.nl (R. Verpoorte).

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described, to prove the thesis that the aroma compounds are present in the green bean as glucosides.

2. Materials and methods

2.1. Materials

The organic solvents used in the reactions were all of p.a. quality and were made water free by drying over 3 Å molecular sieves (Merck, Germany). α-D-Acetobromoglucose, silver trifluoroacetate, penta acetylglucose, piperidine, potassium *t*-butylate and malonic acid were from Fluka, vanillin, vanillic acid, 2-phenylethanol, guaiacol, ferulic acid, sodiumborohydride, *p*-nitrophenyl-β-D-glucopyranoside and polyvinyl polypyrrolidone were from Sigma, *p*-cresol, creosol and *p*-hydroxy benzaldehyde were from Aldrich, pyridine was from Baker.

2.2. Synthesis of glucosides

Reaction yields are indicated in parentheses.

2.2.1. Glucosides of vanillin (GV, 11.2%)/vanillic acid (GVA)/creosol (Gcreo, 20.8%)

• *Glucosylation* method A (Mulkens & Kapetanidis, 1988): Aglycon (5 mmol) was dissolved in 8 ml acetone and 5 ml 1 M KOH. α-D-Acetobromoglucose (10 mmol) in 16 ml acetone was added. If the pH decreased below 8, again 5 ml of 1 M KOH were added. The mixture was stirred for 2 h at room temperature. Product formation was checked on TLC (hexane:ethyl acetate = 1:1, v/v). The products were extracted with dichloromethane, the organic layer was evaporated and the acetylated glucosides were recrystallised from ethanol.

• *Deacetylation* method B (Leong, Uzio, & Derbesy, 1989): Crystals were dissolved in acetone: 1 M NaOH (1:1, v/v). After half an hour at room temperature glucoside formation was checked on TLC (dichloromethane:methanol:water = 78:20:2, v/v/v). The solution was neutralised with Amberlite H^+ (BDH, UK), filtered and the solvent was evaporated. The product was recrystallised from ethanol.

2.2.2. Glucosides of p-cresol (Gp-c, 72.0%)/2-phenylethanol (G2phE, 61.0%)

• *Glucosylation* method C (Kuribayashi, Ohkawa, & Satoh, 1989): Silver trifluoroacetate was dried at 100 °C for 1 h in a vacuum oven before use. An additional amount of 5% was weighed for loss on drying.

• Penta-acetylglucose (0.5 mmol), aglycon (1 mmol) and silver trifluoroacetate (0.75 mmol) were dissolved in dichloromethane (15 ml) under nitrogen and cooled to 0 °C. $SnCl_4$ (1.5 mmol) was added and stirred for 2 h in the absence of light. The formation of products was checked on TLC (petroleum ether:ethyl acetate = 2:1, v/v). The reaction was stopped by addition of 10% aq. $NaHCO_3$ (5 ml) and stirred for a further 20 min. The mixture was filtered using a Buchner funnel, the filtrate was extracted several times with diethyl ether. The combined fractions were washed with brine, dried over $MgSO_4$ and concentrated. Crystals of *p*-cresol tetra-acetylglucoside appeared overnight. 2-Phenylethanol tetra-acetylglucoside was purified on a silica column (dichloromethane:methanol:water = 78:20:2, v/v/v).

• *Deacetylation* method D (Zemplén & Pascu, 1929): The O-glycoside (1 mmol) was dissolved in 5 ml methanol and potassium *t*-butylate (KO^tBu) was added until pH 8–9. The reaction mixture was stirred at room temperature. The reaction was followed on TLC (methanol:ethyl acetate = 5:95, v/v). The reaction terminated within 5 min. The solution was neutralised with Amberlite H^+, filtered and concentrated.

2.2.3. Glucosides of p-hydroxbenzaldehyde (GpOHb, 19.2%)/vanillin (GV, 20.6%)

• *Glucosylation* method E (Leong et al., 1989): Aglycon (10 mmol) was dissolved in 1 M KOH in ethanol (10 mmol). α-D-Acetobromoglucose (12 mmol) in chloroform was added. The mixture was stirred and refluxed for 1 h, then cooled and filtered. Ice water was added and the organic phase was collected. The reaction was followed on TLC (hexane:ethyl acetate = 1:2, v/v). After purification on silica (hexane:ethyl acetate = 1:2, v/v) the product was pure.

• *Deacetylation*: method D.

2.2.4. Glucoside of ferulic acid (GFA, 45.0%)

• *Knoevenagel-synthesis* method F (Hann, 1934): To 0.41 mmol tetraacetylglucovanillin (as described above) 2.5 eq malonic acid, 5 ml pyridine and two drops of piperidine were added. The mixture was refluxed at 100 °C over a steam bath for 1.5 h, then heated to boiling point for a few min and cooled down. Pyridine was evaporated. The acetylated product was recrystallised from 95% ethanol.

• *Deacetylation*: method D. Glucoferulic acid was obtained after recrystallisation in hot water.

2.2.5. Glucoside of vanillyl alcohol (Gvalc)

• *Reduction* (method G): Glucovanillin (0.16 mmol) was dissolved in 5 ml methanol. Fifty milligrams of $NaBH_4$ in 500 μl of 1 M NaOH were added. The reaction was followed on TLC (dichloromethane:methanol:water = 78:20:2, v/v/v). The solution was neutralised with a small quantity of acetic acid and then concentrated.

2.2.6. Glucoside of guaiacol (Gguaia, 18.0%)

• *Glucosylation*: method E. Instead of KOH, NaOH was used.

• *Deacetylation*: method B.

2.3. Identification of glucosides

Structures were confirmed by NMR analysis on a 400 MHz NMR spectrometer (Bruker, Germany) with deuterated methanol as solvent (Table 1). The methanol signal was used to align the spectrum. The structure formulas are depicted in Fig. 1.

2.4. Extraction of glucosides from vanilla beans

Green beans of *Vanilla planifolia* (kindly provided by PT Djasula Wangi, Jakarta, Indonesia), cut into 1 cm pieces, were frozen in liquid nitrogen and then ground in a Waring blender. The powder was extracted for 10 min in 0.1 M acetate buffer pH 5 (1:2 w/v) at 90 °C (Günata, Bayonove, Baumes, & Cordonnier, 1985). After rapid cooling, the extract was filtered over two layers of Miracloth and then centrifuged at 17 000 *g* for 30 min.

The glucosides in the extract were isolated by solid phase extraction on a 35 ml Oasis™ HLB cartridge (6 g of sorbent) (Waters, Etten Leur, The Netherlands). After equilibration of the cartridge with 50 ml methanol followed by 50 ml water, the sample was loaded. Unbound compounds were eluted with 30 ml of water. Glucosides were collected by elution with methanol:water = 1:1 (v/v; 60 ml) and the cartridge was cleaned for further use with methanol.

2.5. Analysis of glucosides

The glucosides were analysed on a Waters 600E HPLC equipped with a Waters 991 Photo Diode Array detector and a Phenomenex ODS Hypersil column (5 μm C18, 2504.6 mm) using a Phenomenex SecurityGuard C18 precolumn. A gradient using solvent A (water:acetonitrile:acetic acid = 95:5:0.75, v/v/v) and solvent B (water:acetonitrile:acetic acid = 5:95:0.75, v/v/v) started with 0% B. In 30 min% B rose to 20%, then in 7.5 min to 80% B. Column wash with 100% B lasted 1.5 min, then the column was equilibrated for the next run with 100%

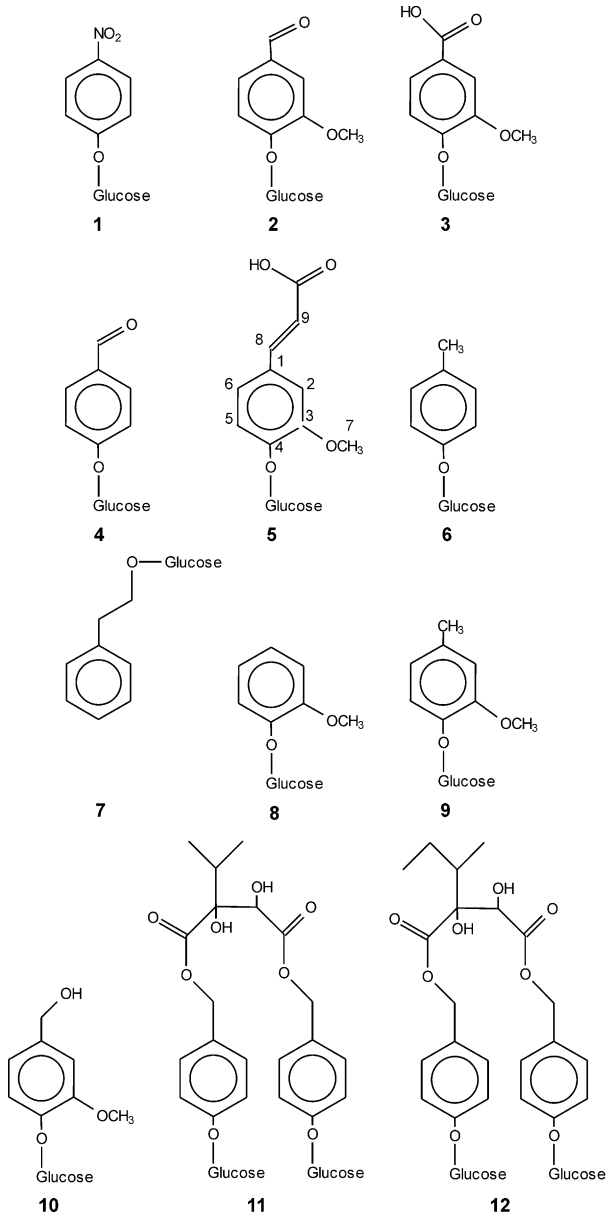

Fig. 1. Structure formulas of β-D-glucopyranosides of (1) *p*-nitrophenol; (2) vanillin; (3) vanillic acid; (4) *p*-hydroxybenzaldehyde; (5) ferulic acid; (6) *p*-cresol; (7) 2-phenylethanol; (8) guaiacol; (9) creosol; (10) vanillyl alcohol; (11) glucoside A; and (12) glucoside B.

Table 1
^{1}H-NMR data (ppm) of the aglycone moiety of several synthetic β-D-glucosides and the anomeric proton*

H	GV	GpOHb	GFA	Gp-c	G2phE	Gguaia	Gcreo	GValc
1	–	–	–	–	–	6.84	–	–
2	7.47	7.80 *d*, $J_{2,3}$ *8.86*	7.01	7.07 *d*, $J_{2,3}$ *8.76*	7.16	6.94	6.81	7.04
3	–	7.16 *d*, $J_{3,2}$ *8.80*	–	6.97 *d*, $J_{3,2}$ *8.64*	7.23	–	–	–
4	–	–	–	–	7.23	–	–	–
5	7.25 *d*, $J_{5,6}$ *8.21*	7.16 *d*, $J_{5,6}$ *8.80*	7.01	6.97 *d*, $J_{5,6}$ *8.64*	7.23	7.07 *d*, $J_{5,6}$ *7.65*	7.03 *d*, $J_{5,6}$ *8.16*	6.89 *d*, $J_{5,6}$ *8.25*
6	7.51 *m*	7.80 *d*, $J_{6,5}$ *8.86*	7.01	7.07 *d*, $J_{6,5}$ *8.76*	7.16	6.94	6.69 *d*, $J_{6,5}$ *8.18*	7.15 *d*, $J_{6,5}$ *8.24*
7	3.96	–	3.82	–	–	3.74	3.82	3.88
8	9.86	9.67	7.51 *d*, $J_{8,9}$ *15.93*	2.26	2.92 *t*, $J_{8,9}$ *1.64*	–	2.27	4.55
9	–	–	6.22 *d*, $J_{9,8}$ *15.93*	–	3.22 m	–	–	–
*1′	5.03 *d*, $J_{1'\text{-}2'}$ *7.53*	5.17 *d*, $J_{1'\text{-}2'}$ *7.51*	4.81 *d*, $J_{1'\text{-}2'}$ *7.32*	4.83 *d*	4.31 *d*, $J_{1'\text{-}2'}$ *7.76*	4.88 *d*, $J_{1'\text{-}2'}$ *7.53*	4.81 *d*, $J_{1'\text{-}2'}$ *7.49*	4.89 *d*, $J_{1'\text{-}2'}$ *7.58*

Coupling constants (Hz) are given in italics. Numbering as in Fig. 1.

A. The flow rate was 1 ml min^{-1}. The injection volume was 20 μl.

2.6. Fractionation of glucosides

The same HPLC was used for semi-preparative fractionation of glucosides using a μBondapak C18 column (300×7.8 mm; Waters, USA). A gradient using solvent A (water:acetonitrile:acetic acid = 95:5:0.75, v/v/v) and solvent B (water:acetonitrile:acetic acid = 5:95:0.75, v/v/v) started with 0% B. In 30 min% B rose to 10%, then in 7.5 min to 80% B. Column wash with 100% B lasted 1.5 min, then the column was equilibrated for the next run with 100% A. Flow rate was 2.5 ml min^{-1}, injection volume was 100 μl. Fractions of 2.5 ml were collected. After vacuum evaporation of the eluents, the residue was dissolved in solvent A and analysed on analytical HPLC, using the method described above.

2.7. Enzyme extraction

Green vanilla beans were harvested in July 1999, in Indonesia and arrived in the Netherlands within 5 days without further preservation. Samples were extracted with 0.15 M BisTris-Propane (BTP) buffer pH 8 (Sigma, St. Louis, MO). Extraction was performed by adding buffer (supplemented with 2 mM EDTA and 3 mM dithiothreitol) to the beans which were cut into 1-cm pieces (1:1 v/w). 5% w/w polyvinyl polypyrrolidone (PVPP) was added. The mixture was ground in a Waring blender, centrifuged and then stored at −80 °C in the presence of 15% glycerol until analysis (Dignum, Kerler, & Verpoorte, 2001). Interfering compounds (aglycons, sugars) were removed using a PD-10 column (Pharmacia, Sweden) before further analysis.

2.8. Enzyme assay

The kinetics of β-glucosidase activity were assayed towards *p*-nitrophenyl-β-D-glucopyranoside (pnpG) and eight glucosides naturally occurring in vanilla beans (GV, GVA, GFA, GpOHb, Gp-c, Gcreo, Gguaia, G2phE). The glucosides were dissolved in McIlvaine buffer pH 7.0 in the range of 0.25–30 mM. An amount of 190 μl was incubated with 10 μl of enzyme extract for exactly 60 min at 30 °C and then heated at 100 °C for 10 min in a closed vial to inactivate the enzyme. Analysis of the formed aglycons was performed on a Waters 600E HPLC as described in the Analysis of glucosides section.

The kinetic parameters V_{max} and K_m were calculated using the aglycon peak areas at 280 nm. Calibration curves of aglycons were also measured at 280 nm.

3. Results and discussion

3.1. Identification of glucosides

Based on the results of GC-Olfactometry of a cured bean extract, it was shown that some compounds that were present in low concentrations, have a high impact on the overall vanilla flavour (results not shown). These compounds were identified as *p*-cresol, 2-phenylethanol, guaiacol and creosol. To investigate whether these compounds are present as glucosides in the green beans, their glucosides were synthesised. Besides these compounds, other compounds of interest were glucosylated as well (GV, GVA, GFA, GpOHb, Gp-c, Gcreo, Gguaia, G2phE, GValc). The synthesis of these glucosidic compounds required quite different methods, there was no single method found for glucosylation of all compounds.

After preparative HPLC fractionation, four glucosides could be identified in a green bean extract by means of NMR. These are GV, GpOHb and bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate and bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2-butyl)tartrate [glucoside A and B respectively, as described by Kanisawa (1993)].

Further glucosides have been identified by comparing the HPLC retention time and UV-spectra of the compounds in the extract, with those of the synthesised reference compounds. Samples were also spiked with the synthesised reference compounds. The compounds that were present in high amounts were identified as GV, Gvalc, GVA, GpOHb, Glucoside A and B (Fig. 2a).

The minor compounds of interest (Gp-c, Gcreo, Gguaia, G2phE) could not be detected with this method, maybe because their concentration was too low. From the kinetics experiments (as shown below), it was concluded that these compounds are not hydrolysed by the vanilla β-glucosidase. Therefore an incubation of the glucoside extract with the vanilla β-glucosidase was done. After SPE on an Oasis™ HLB cartridge, the extract was enriched in some minor glucosides. From the chromatogram of this mixture it was clear that Gp-c and Gcreo were present (Fig. 2b). To ensure that these compounds were indeed glucosides, the extract was digested with almond β-glucosidase and with 0.1 M HCl. In incubations with synthetic glucosides, it was shown that the glucosides were hydrolysed with these substances. After treatment of the glucoside extract, the peaks disappeared in the chromatogram, further confirming that Gp-c and Gcreo were present as minor compounds in green vanilla beans. Kanisawa (1993) postulated the presence of Gcreo, but it was based on the identifiacation of the aglycon after hydrolysis with β-glucosidase. He did not detect the glucoside itself.

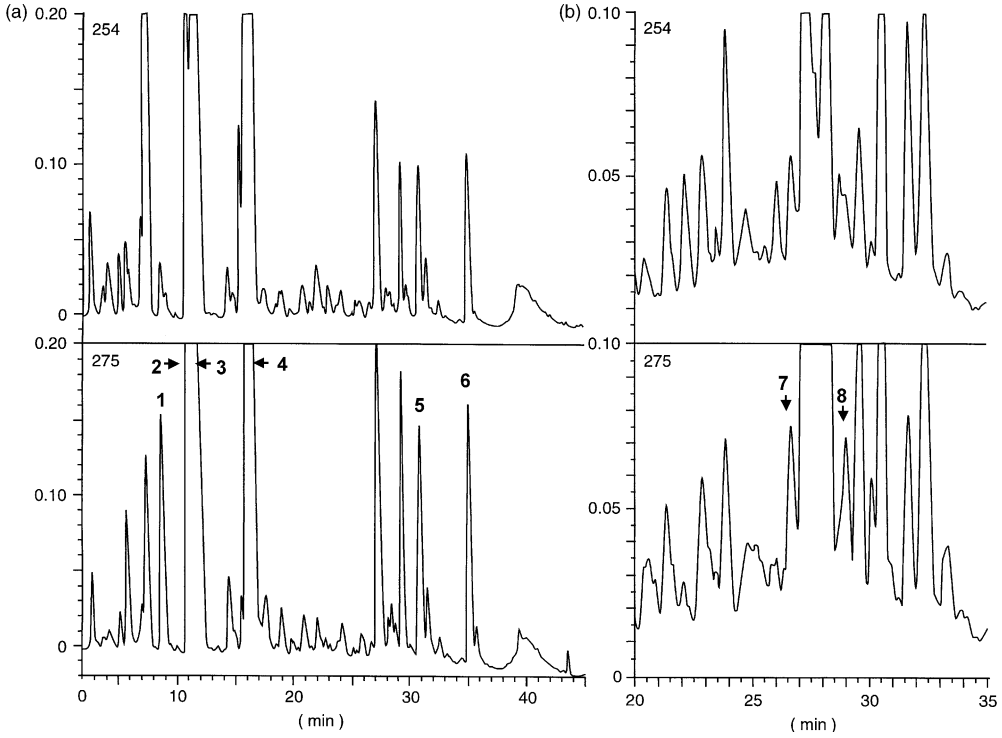

Fig. 2. (a) HPLC-chromatogram of a green vanilla bean glucoside extract at 254 nm (upper) and 275 nm (lower): (1) GValc; (2) GpOHb; (3) GVA; (4) GV; (5) Glucoside A; (6) Glucoside B. (b) HPLC-chromatogram at 254 nm (upper) and 275 nm (lower), showing minor glucosides Gp-c (7) and Gcreo (8), after hydrolysis with vanilla β-glucosidase.

The glucosides G2phE, Gguaia and GFA were not identified in the vanilla bean in this study. Either their concentration in the extract was too low, or the aglycons identified in cured vanilla aroma, were not derived from glucosides. The fact that Gguaia is identified in an earlier study (Dignum et al., in press), might indicate that the concentration of the minor glucosides may vary and is sometimes too low to identify them in the extract. This can be caused by the difference in ripeness or differences in the cultivars used.

3.2. Enzyme kinetics experiments

The kinetic parameters of β-glucosidase activity in a green vanilla bean extract were investigated. These experiments were performed on β-D-glucosides of *p*-nitrophenol, vanillin, vanillic acid, *p*-hydroxy benzaldehyde, ferulic acid, *p*-cresol, 2-phenylethanol, guaiacol and creosol (Fig. 1). Initially, an attempt was made to express the results based on the decrease of the substrate by means of HPLC. This seemed to be the most correct approach, because the released aglycon can also be oxidised or reduced and therefore the measured value may be too low. However, the decrease in peak area of the glucoside was too small to be accurate. At higher concentrations, the values for peak area were outside the linear part of the calibration curve. Therefore, the increase of the aglycon peak was measured. The calibration curves of all aglycons tested, were linear in the range of 0.25–5 mM.

The relation between incubation time and product formation was investigated. It showed that incubation with 1 mM of pnpG resulted in a linear plot between 10 and 60 min. When the incubation was done with 15 mM of pnpG, the results were also linear after incubation with 0.06 till 1.0 μg protein. Based on these results it was decided that the incubations would be performed with 0.6 μg protein for 60 min in the presence of 0.25–30 mM glucosides.

For all glucosides, exept Gp-c and G2phE, the assays resulted in the typical Michaelis–Menten plot. This indicates that the chosen method using the released aglycons, is accurate enough for these assays. In the assays of glucoside Gp-c and G2phE, there was no visible formation of aglycon. Table 2 shows that the

Table 2
Kinetic parameters of β-glucosidase activity from vanilla for several glucosidic compounds

Glucoside of	V_{max} (IU mg^{-1} protein)	K_m (mM)
p-Nitrophenol	11.5	3.3
Vanillin	9.4	5.1
Vanillic acid	3.1	21.3
p-Hydroxy benzaldehyde	10.6	6.9
Ferulic acid	4.9	5.3
p-Cresol	–	–
2-Phenylethanol	–	–
Guaiacol	15.9	131.8
Creosol	6.7	29.4

Table 3
Kinetic parameters of some β-glucosidase activities from different sources towards *p*-nitrophenol-β-D-glucoside

Name	Origin	K_m (mM)	V_{max}	Ref.[a]
Vanilla (*Vanilla planifolia* Andrews)	Monocot	0.38	50 000 IU	1
Oat (*Avena sativa* L.) Type I	Monocot	3.12	20 000 IU mg^{-1} prot	2
Oat (*Avena sativa* L.) Type II	Monocot	1.0	3300 IU mg^{-1} prot	2
Maize (*Zea mays* L.)	Monocot	2.96		3
Barley (*Hordeum vulgare* 'Gerbel')	Monocot	1.8		4
Narcissus (*Narcissus papyraceus* 'Ziva')	Monocot	0.083	25.9 IU mg^{-1} prot	5
Carrot (*Daucus carota* L.)	Dicot	0.12	2170 IU mg^{-1} prot	6
Thai rosewood (*Dalbergia cochinchinensis* Pierre ex Laness.)	Dicot	5.4		7
Wooly foxglove (*Digitalis lanata* Ehrh.)	Dicot	10.5	0.3 IU mg^{-1} prot	8
Almond [*Prunus dulcis* (Mill.)D.Webb]	Dicot	3.6		9
Cassava (*Manihot esculenta* Crantz)	Dicot	0.36		10
Sweet orange (*Citrus sinensis* 'Valencia')	Dicot	0.115	0.32 IU mg^{-1} prot	11
Kluyveromyces marxianus IMB3	Yeast	1.1		12
Candida sake	Yeast	6.9		13
Micromonospora chalcae	Bacteria	0.25		14
Micromonospora chalcae (expressed in E. Coli)	Bacteria	0.19		15
Cellulomonas biazotea	Bacteria	0.025	4.8 IU	16
Nectria catalinensis	Fungus	0.25	0.23 IU mg^{-1} prot	17

[a] 1. Hanum (1997); 2. Kim, Song, and Kim (1996); 3. Oikawa, Ebisui, Sue, Ishihara, and Iwamura (1999); 4. Dietz, Sauter, Wichert, Messdaghi, and Hartung (2000); 5. Reuveni, Zohar, Evnor, and Hetzroni (1999); 6. Konno, Yamasaki, and Katoh (1996); 7. Srisomsap et al. (1996); 8. May, and Kreis (1997); 9. Fischer, and Peissker (1998); 10. Keresztessy, Brown, Dunn, and Hughes (2001); 11. Cameron, Manthey, Baker, and Grohmann (2001); 12. Barron, Marchant, McHale, and McHale (1995); 13. Gueguen, Chemardin, and Arnaud (2001); 14. Gallagher, Winters, Barron, McHale, and McHale (1996); 15. Winters, Gallagher, Barron, Rollan, and McHale (1996); 16. Lau, and Wong (2001); 17. Pardo, and Forchiassin (1999).

K_m-value for pnpG compared to other major glucosides is in the same range in the case of GV, GFA and GpOHb. For the minor glucosides, the K_m-value was much higher indicating less affinity of the enzyme for these substrates. There was no clear preference for the naturally occurring glucosides compared to the synthetic glucoside pnpG. The highest V_{max} was found for glucosides pnpG, GV, GpOHb and Gguaia. In an earlier study (Dignum et al., in press), it was shown that the minor aroma compounds were formed in green beans with totally inactivated β-glucosidase. This finding, combined with the low affinity of the enzyme to the minor glucosides supports the theory that the degradation of glucosides in vanilla curing may not be completely enzymatic.

In Table 3, a comparison is made between the kinetic parameters of β-glucosidases from several sources found in recent literature and β-glucosidase activity from vanilla. If previously reported results for vanilla are compared with the result from the present research, it can be concluded that the latter has a ten times higher K_m-value for pnpG. If β-glucosidases from other sources are compared (Table 3), it shows that the K_m varies from 0.083 to 10.5 mM. The K_m-value found for vanilla in this investigation (3.3 mM for pnpG) is in the same range, as can be expected for a β-glucosidase. The values of V_{max} vary from 0.23 to 20000 μmol min^{-1} mg^{-1} protein from different sources.

The β-glucosidases from many different plants (Table 3) are able to hydrolyse the synthetic substrate pnpG, even better than the natural occurring substrates in some cases. In this study, Gp-c and G2phE were not hydrolysed at all and Gcreo and Gguaia only with a very high K_m-value (>30 mM). This implies that it is always important not just to look at the results of a general glucosidase activity, using pnpG as a substrate, but also to carry out kinetic experiments with endogenous plant compounds.

If the structures of the glucosides under investigation are compared (Fig. 1), it can be concluded that the enzyme has the highest affinity for a glucoside with a polar group in the *para*-position (compounds **1**–**5**). Glucosides with an apolar or no substituent in the *para*-position and a methoxyl-group in *ortho*-position are hydrolysed poorly (compounds **8** and **9**). Compounds lacking a polar group in the *para*-position and a methoxyl-group in *ortho*-position (**6** and **7**) or that have the glucose attached to the sidechain (**7**) are not hydrolysed at all.

4. Conclusion

The presence of a series of phenolic glucosides could be confirmed by comparison with synthesised reference compounds. The crude β-glucosidase isolated from the green beans, clearly shows selectivity towards these glucosides, some are even not accepted as a substrate. The presence of the comparing aglucons in cured beans thus points to the presence of these compounds as such or to chemically control;ed production in the curing proces.

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