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To cite this version:

Jean Marc Brillouet, Eric Odoux. In vivo kinetics of beta-glucosidase towards glucovanillin and related phenolic glucosides in heat-treated vanilla pod ([i]Vanilla planifolia[/i], Orchidaceae). Fruits, 2010, 65 (2), pp.85-95. 10.1051/fruits/20010004 . hal-01223561

HAL Id: hal-01223561

https://hal.science/hal-01223561v1

Submitted on 2 Nov 2015

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Original article

In vivo kinetics of β-glucosidase towards glucovanillin and related phenolic glucosides in heat-treated vanilla pod (*Vanilla planifolia*, Orchidaceae)

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Received 28 September 2009
Accepted 3 November 2009

Fruits, 2010, vol. 65, p. 85–95
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DOI: 10.1051/fruits/20010004
www.fruits-journal.org

Resumen Español, p. 95

In vivo kinetics of β-glucosidase towards glucovanillin and related phenolic glucosides in heat-treated vanilla pod (*Vanilla planifolia*, Orchidaceae).

Abstract — Introduction. The traditional curing of vanilla pods includes "killing" and sweating steps when pods are exposed to heat (35–65 °C) for various lengths of time. Although it is known that liberation of vanillin and other phenolics from their non-aromatic glucosides is due to the action of an endogenous β-glucosidase, its *in vivo* kinetics remained unknown. **Materials and methods**. Mature green vanilla pods were pretreated for 2 h at 50 °C, 55 °C and 60 °C, then stored for 118 days at 27 °C. Phenolic glucosides and their aglycons were extracted at regular intervals during the storage period and analyzed by HPLC. **Results and discussion**. All phenolic β-glucosides were slowly hydrolyzed during the storage period with production of vanillin, *p*-hydroxybenzaldehyde, vanillic acid, and other unknown aglycons. Most of the β-glucosidase was heat-denatured by the pretreatment, and analysis of its kinetic parameters showed that it adopts, *in vivo*, an allosteric mode of functioning with a lower affinity for glucovanillin than *in vitro*, where it behaves as a Michaelian enzyme. **Conclusion**. Extensive research is needed to confirm the allosteric mechanism of the vanilla β-glucosidase *in vivo*.

France / *Vanilla planifolia* / vanilla / processing / heat / glucovanillin / β-glucosidase / affinity constant / allostery

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Cinétique de fonctionnement *in vivo* d'une β-glucosidase vers la libération de la glucovanillin et de glucosides phénoliques connexes dans la gousse de vanille soumise à un traitement thermique (*Vanilla planifolia*, Orchidaceae).

Résumé — Introduction. Le procédé traditionnel de préparation des gousses de vanille comprend une étape d'échaudage et une d'étuvage pendant lesquelles les gousses sont soumises à la chaleur (35–65 °C) pendant des durées variables. Bien que l'on sache que la libération de la vanilline et autres composés phénoliques à partir de leurs glucosides non aromatiques est due à l'action d'une β-glucosidase endogène, ses paramètres cinétiques de fonctionnement *in vivo* ne sont pas connus. **Matériel et méthodes**. Des gousses vertes matures ont tout d'abord été traitées pendant 2 h à, respectivement, 50 °C, 55 °C et 60 °C, puis stockées pendant 118 jours à 27 °C. Les glucosides de phénols et leurs aglycones ont été extraits à intervalles réguliers pendant la période de stockage et analysés par HPLC. **Résultats et discussion**. Tous les glucosides de phénols ont été lentement hydrolysés pendant la période de stockage avec libération de vanilline, *p*-hydroxybenzaldéhyde, acide vanillique et autres aglycones inconnus. L'essentiel de l'activité β-glucosidase a été dénaturé par la chaleur pendant le traitement préliminaire et l'analyse de ses paramètres cinétiques a montré que l'enzyme adopte, *in vivo*, un mode de fonctionnement allostérique avec une affinité pour la glucovanilline plus faible qu'*in vitro* où elle se comporte comme une enzyme michaélienne. **Conclusion.** La confirmation du mécanisme allostérique de la β-glucosidase *in vivo* de vanille requiert une recherche approfondie.

France / *Vanilla planifolia* / vanille / traitement / chaleur / glucovanilline / β-glucosidase / constante d'affinité / allostérie

Article published by EDP Sciences and available at http://www.fruits-journal.org or http://dx.doi.org/10.1051/fruits/20010004

1. Introduction

The vanilla pod, a berry which turns into a capsule upon drying [1–3], is a generator of a highly prized aroma; it owns its full enzyme-substrate equipment, made up of a β-glucosidase [4, 5] and phenolic β-D-glucosides [6] which, under certain conditions, are brought together with subsequent hydrolytic release of flavoring aglycons, mostly vanillin [2, 7–12]. Anatomical distributions of the β-glucosidase and its substrates have recently been extensively described [3, 4, 13, 14].

Vanilla pods are harvested at the green mature stage, lacking at that time the characteristic vanilla flavor; further processing of the pods (the traditional "curing") includes four steps: "killing" (or scalding), sweating, drying and conditioning [8, 9, 15]. The first "killing" phase, when pods are dipped in hot water (typically 50–60 °C, 3 min) [15], aims at arresting the vegetative development of the pod, *i.e.*, to prevent the dehiscence detrimental to the final quality [8]; during this later and subsequent phases, multiple biological, chemical and physical events happen (hydrolysis, oxidation, water loss, migration, crystallization and sublimation of substances, microbial development, etc.) which in the end result in semi-dry vanilla pods of various quality attributes. Numerous studies have been devoted to elucidation of the various mechanisms involved [2, 7, 9–12,16–18], the first crucial event being the hydrolysis of phenolic β-glucosides by a β-glucosidase: Odoux *et al.* [5] purified and characterized a vanilla β-glucosidase, while Dignum *et al.* [10] studied the *in vitro* kinetics of a crude β-glucosidase on several vanilla β-glucosides. However, the *in vivo* mode of action of this β-glucosidase activity is still not fully understood: it has been suggested, for instance, that the emergence of certain aroma compounds in heat-treated pods (*e.g.*, *p*-cresol) would not result from an enzymatic breakdown of their glucosides [11], although *p*-cresol β-glucoside has been described by Kanisawa [6], and its aglycon is a constituent of the vanilla flavor; also, the same authors suggested, since the β-glucosidase activity, non-specifically measured on *p*-nitrophenyl β-D-glucopyranoside, is no longer measurable after "killing" and since nevertheless β-glucosides slowly disappear from the pods, the existence of another β-glucosidase "specific" to glucovanillin which would somewhat resist the heat treatment. The existence of two β-glucosidases in the pod, one of them being "specific" to glucovanillin, had been previously mentioned by Kanisawa [19] but its reality had never been formally confirmed.

Figure 1.
Green mature vanilla pods.

Márquez and Waliszewski studied the *in vitro* kinetics of thermal inactivation of a vanilla β-glucosidase crude extract [12]. However, and to our knowledge, no results were ever reported on the *in vivo* kinetics of this enzyme. Since the behavior of an enzyme might differ noticeably in a test tube assay (*in vitro*) and in its natural environment (*in vivo*), the aim of our work was thus to describe in detail kinetics of the β-glucosidase on vanilla pod β-glucosides in heat-treated vanilla pods (*in vivo*).

2. Materials and methods

2.1. Materials

Sound mature green vanilla pods were harvested 8 months after pollination (Réunion Island, France) (*figure 1*).

Vanillin, vanillic acid, *p*-hydroxybenzaldehyde, α-D-acetobromoglucose and *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) were from Fluka (Buchs, Switzerland). All other chemicals and solvents were of the highest available quality. Synthesis of glucosides was achieved according to Dignum *et al.* [10].

2.2. Heat treatment of vanilla pods

For heat treatment, the middle region of vanilla pods was transversally sliced into twelve adjacent sections (plus one located in the middle of the pod which will be used for β-glucosidase measurements) (~3 mm thickness) and slices were sealed in a polyethylene bag and placed for 2 h in a temperature-controlled water bath (50 °C, 55 °C and 60 °C, respectively). Slices were then removed and kept at 27 °C for 118 days, one slice being withdrawn and frozen every ~10 days. They were then analyzed for their β-glucosidase activity and phenolic contents.

2.3. Extraction and determination of glucovanillin and related phenolics

Extraction and determination of glucovanillin and related phenolics were performed according to Odoux and Brillouet [3]: briefly, sections (~150 mg each) were extracted by [methanol:water] (50:50, v/v); after centrifugation, the supernatant was then submitted to quantitative analysis by HPLC separation of phenolics and their glycoconjugates on a (250 mm × 4.6 mm) Modulocart QSLichrospher 5-m ODS2 column operated at 0.5 mL·min^{-1} and 30 °C. The mobile phase consisted of [water:formic acid] (98:2, v/v) (eluant A) and [water:acetonitrile:formic acid] (18:78:2, v/v/v) (eluant B). The elution program was as follows: 8–13% eluant B (0–10 min); 13–20% eluant B (10–30 min); 20–8% eluant B (30–35 min). The column effluent was monitored at 280 nm. Triplicate samples were injected at a level of 10 µL. External standardization was achieved with pure glucovanillin (ChromaDex, Irvine, CA), synthetic glucosides and commercial aglycons. Data were expressed either as mM in the water phase of the pods, *i.e.*, water content 85% (kinetic study), or % of fresh weight.

2.4. Extraction and measurement of the β-glucosidase activity

After heat treatment (*i.e.,* at time zero of the storage period), withdrawn pod sections (~150 mg, central region) were dilacerated at ambient temperature in 50 mL of 0.1 M phosphate buffer (pH 7.0). After centrifugation (14 000 *g*, 5 min, 20 °C), the filtrate was diluted 10 × with the same buffer.

Activity was measured as described in Odoux *et al.* [5]: briefly, an aliquot of enzyme extract was added to 200 µL of 4 mM *p*-nitrophenyl β-D-glucopyranoside in the above phosphate buffer and the total volume was made up to 400 µL with phosphate buffer. After 20 min of incubation at 40 °C, 1 mL of 0.5 M NaOH was added and the optical density was read at λ = 400 nm. Activity was also measured on glucovanillin [5]. It was expressed as nanokatals, *i.e.*, 1 nKat being the amount of enzyme hydrolyzing 1 nanomole of substrate per sec.

2.5. Purification of the vanilla β-glucosidase

Purification of the vanilla β-glucosidase was performed from 1 kg of mature green vanilla pods (~8 months after pollination) according to Odoux *et al.* [5].

3. Results

3.1. Kinetics of the purified vanilla β-glucosidase

We formerly published the purification and characterization of the vanilla β-glucosidase [5] and gave its K_m on *p*-nitrophenyl β-glucoside and glucovanillin. Having purified higher quantities of this enzyme for a future preparation of antibodies, we again measured these constants with twelve concentrations of each substrate: an excellent linearity of the reciprocal 1/v = f (1/s) was obtained (*figure 2*). Corrected values are: K_m = 4.3 ± 0.1 mM on *p*-nitrophenyl β-glucoside, and K_m = 5.0 ± 0.2 mM on glucovanillin.

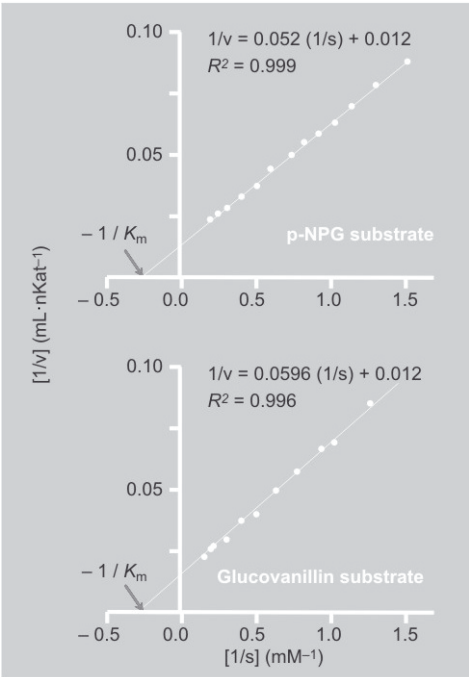

Figure 2.
Reciprocal representation [1/v = f (1/s)] for β-glucosidase with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) and glucovanillin as substrates.

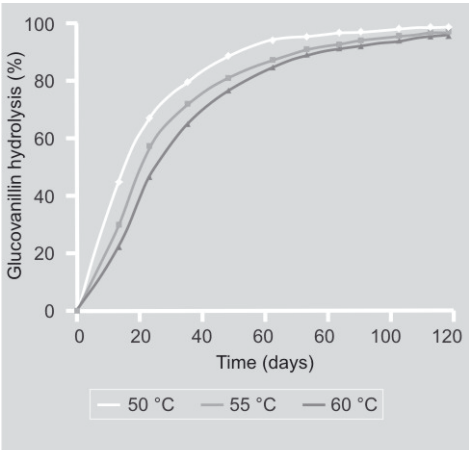

Figure 3.
Time curves of glucovanillin hydrolysis in heat-treated vanilla sections.

3.2. Fate of phenolics and the β-glucosidase after heat treatment

It must first be noted that the slice water content did not change during the heat pretreatment and subsequent storage, since they were stored in sealed gas- and waterproof polyethylene bags; this was checked on separate experiments. No mold development or microbial alteration were detected either. The starting green mature pods contained (130 ± 7) mM glucovanillin.

As previously mentioned [9, 20], no β-glucosidase activity was measurable after the preliminary heat treatment, even on undiluted extracts (data not shown).

We thus measured the disappearance of glucovanillin, its substrate, during the storage period (*figure 3*). Although hydrolysis started during the preliminary heat treatment [20], the beginning of hydrolysis was taken to be at the end of this treatment, *i.e.*, at time zero of storage; indeed, for calculation of the affinity constant for glucovanillin, one must have identical conditions of temperature in time (*i.e.*, 27 °C). As expected [8], its concentration decreased slowly over time, reaching a very low level after 118 days, and the rate of hydrolysis (v_{hyd}) was clearly related during the first 13 days of storage to the temperature of the preliminary 2-h heat treatment, *i.e.*, $v_{hyd(50\,°C)} > v_{hyd(55\,°C)} > v_{hyd(60\,°C)}$; during this period, since the glucovanillin concentration was high (~50–100 mM), the reaction rate was zero-order with time, *i.e.*, maximum velocity V_{max}. Although the terminal percentages of hydrolysis were similar (~96–98%), the final glucovanillin concentration was also related to the temperature pretreatment, *i.e.*, the higher this temperature, the higher the residual glucovanillin (~4% of initial amount), and reciprocally (~2%); this suggests that a residual non-measurable amount of β-glucosidase activity was at work.

Starting from the hydrolysis curve obtained for 55 °C heat treatment, the first derivative was obtained, giving the velocity of reaction [*v*] at different moments where the concentration of glucovanillin [glcva] was known (*figure 4*). The reaction velocity was then expressed as variation in glucovanillin: 1 unit of millimolarity (mM) in the tissues per day, millimolarity of glucovanillin calculated as the amount of millimoles present in a given volume of the water phase present in the pods (water content ~85%). One can see a typical sigmoid curve where, in the terminal phase of storage when the residual glucovanillin was very low, the reaction rate did not vary linearly with substrate concentration as in the Michaelian model; when glucovanillin was high (initial phase of the storage), the reaction velocity was constant (zero-order). If one admits that the amount of residual enzyme did not

change during the storage period at 27 °C, one has to consider *figure 4* as a typical representation of an allosteric mode of action [21] of the vanilla β-glucosidase, a tetramer [5]; this is also evidenced in the Hanes-Woolf plot and $K_{0.5}$ was taken from a linear interpolation between 28 mM and 100 mM [glcva]: it was 21.8 mM. Thus, the affinity of the β-glucosidase for glucovanillin was decreased in its natural decompartmented medium [11].Then, we built the following Hill plot (*figure 4*): $v = V_m . [glcva]^n / (K_{0.5}^n + [glcva]^n)$, with various n values. Finally, n = 2.9 provided the best fit to the experimental curve.

We measured K_m of the purified enzyme on glucovanillin in conditions mimicking the decompartmented vanilla pod tissue, *i.e.*, 100 mM acetate buffer (pH 5.2) containing 150 mM glucose (*i.e.*, pod sugar content ~2.7%) [3]: in these conditions, its value remained the same as at pH 7.0 in a sugar-free medium, *i.e.*, 5.0 mM [this work], and no allosteric behavior was visible in these dilute conditions.

We obtained two typical HPLC chromatograms of the extraction media from slices at 0 and 118 days of storage (*figure 5*), UV spectra of the detected peaks (*figure 6*), and their kinetics of appearance-disappearance (*figure 7*); compounds decreasing with time will be referred to as "β-glucosides" due to the action of the β-glucosidase, while those increasing will be named "aglycons" resulting from hydrolysis. Compared with other plant extracts, one must first say that the chromatograms were rather poor in number of peaks observed: indeed, no derivatives of flavonol glucosides or flavan-3-ols were detected. At the beginning of the storage, one can see essentially eight peaks (*figure 5*): an unknown β-glucoside (n° 1, λ_{max} = 275 nm), vanillic acid β-glucoside (n° 2, λ_{max} = 254 nm, 291 nm), *p*-OHbenzaldehyde β-glucoside (n° 3, λ_{max} = 268 nm), glucovanillin (n° 5, λ_{max} = 273 nm, 307 nm), *p*-OHbenzaldehyde (n° 11, λ_{max} = 284 nm), an unknown β-glucoside (n° 12, λ_{max} = 271 nm, 308 nm), vanillin (n° 13, λ_{max} = 280 nm, 309 nm), and an unknown (n° 14, λ_{max} = 276 nm, 303 nm). Indeed, after the heat treatment when hydrolysis began, some aglycons had already been released

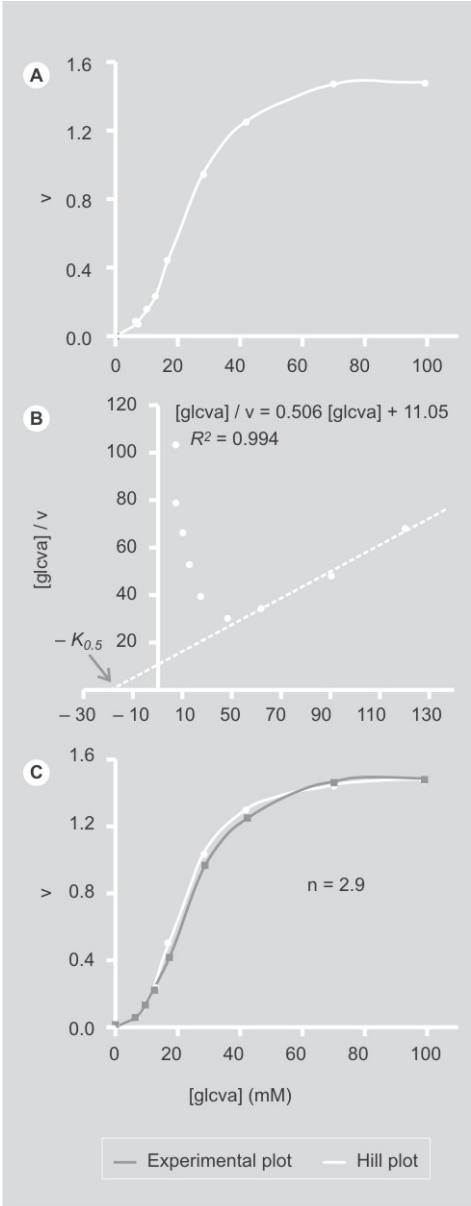

Figure 4.
Direct (A) and Hanes-Woolf (B) plots of reaction rate with glucovanillin concentration (heat pretreatment at 55 °C). In (C) they are compared in experimental and Hill plots.

(peaks n° 11, 13); moreover, in native pods, minor quantities of *p*-OHbenzaldehyde and vanillin are always present aside from the glucosides [8]. At time 118 days, fourteen peaks were observed (*figure 5*); the eight above, plus four unknown aglycons (n° 4, λ_{max} = 271 nm, 307 nm; n° 6, λ_{max} = 271 nm, 307 nm; n° 7, λ_{max} = 271 nm, 306 nm; n° 8, λ_{max} = 279 nm, 309 nm), vanillic acid (n° 9, λ_{max} = 260 nm, 292 nm), and a trace of a hydroxycinnamic acid derivative (n° 10,

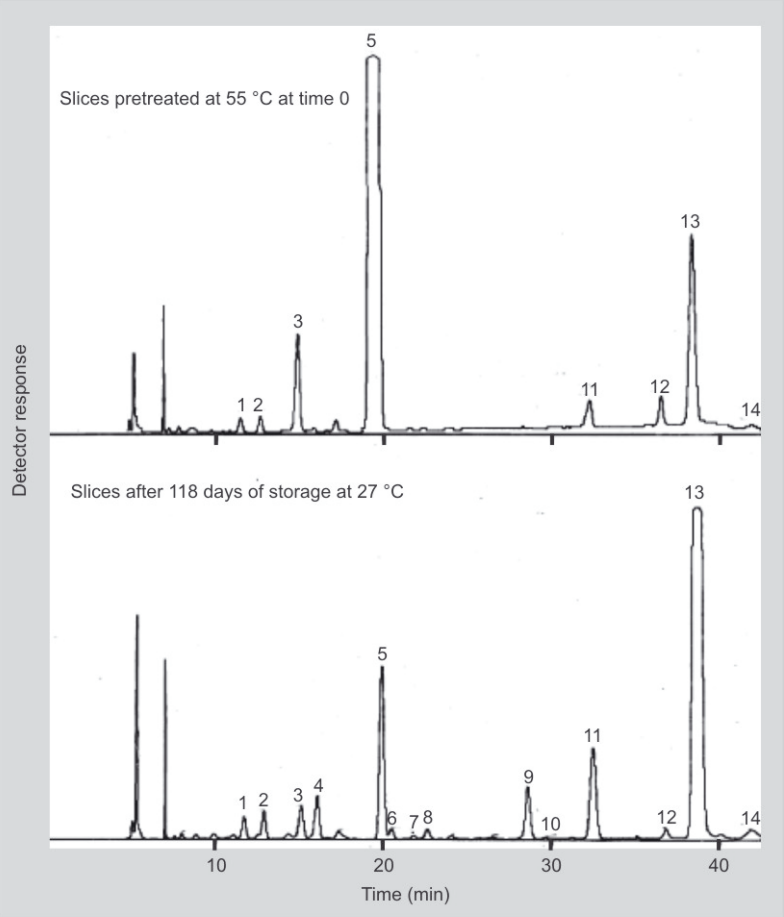

Figure 5.
HPLC chromatograms of the extraction media from different vanilla pod slices pretreated at 55 °C at times 0 and 118 days of storage at 27 °C. Identities of peaks as follows:
1 unknown glucoside,
2: vanillic acid glucoside,
3: *p*-OHbenzaldehyde glucoside, 4: unknown aglycon, 5: glucovanillin,
6: unknown aglycon,
7: unknown aglycon,
8: unknown aglycon, 9: vanillic acid, 10: a hydroxycinnamic acid derivative,
11: *p*-OHbenzaldehyde,
12: unknown glucoside,
13: vanillin, 14: unknown
(enlargements of chromatograms not comparable).

λ_{max} = 298 nm sh, 329 nm). One must say that, apart from peaks n° 4, 9, 11 and 13, other constituents were present at very low levels; however, they might be important for their contribution in the final vanilla flavor [22].

Apart from vanillic acid β-glucoside, glucovanillin and their aglycons, a glucoside (n° 12), and four aglycons (n° 4, 6, 7, 8) exhibited typical spectra similar to glucovanillin. According to its late elution time, peak n° 12 must bear an aglycon of low polarity and could be, according to its late elution time, glucoside A or B [6, 10].

If one examines the curves of appearance-disappearance (*figure 7*), one can see that glucovanillin was almost fully hydrolyzed after 118 days at 27 °C (*figure 3*). Its aglycon, vanillin, reached a plateau corresponding to about one-third of the vanillin liberated from glucovanillin; the same observation is made for *p*-OHbenzaldehyde β-glucoside and its aglycon. Another unknown aglycon (peak n° 4) behaves similarly; however, its glucoside was not seen in the chromatograms (possible undetected coelution with another peak) and we were thus not able to calculate its hydrolysis percent. Two unknown aglycons (n° 6, 7) behave specifically: they were produced up to a maximum at 23–35 days of storage, then decreased strongly until the end; due to their very low concentrations, their glucosides were not detected.

Other curves of hydrolysis clearly show that, *e.g.*, vanillic acid β-glucoside (n° 2) was hydrolyzed more slowly with a linear mode (~50% in 118 days), due to a much lower starting concentration compared with glucovanillin; its aglycon, vanillic acid (n° 9), was released linearly and did not reach a plateau, like the unknown aglycon n° 8. The unknown glucoside (n° 1) behaves the same (~50% hydrolysis in 118 days), and its aglycon was not observed in the chromatograms (possibly hidden in the vanillin peak [10]).

4. Discussion

After having enumerated the numerous possible alterations occurring during and after heat treatment of a vanilla pod, we will discuss our data with regards to them and the literature.

Heat treatment of the pods generates multiple events, most of them being only hypothetical and unsupported (except those with references):

– decompartmentation in the symplasm with disruption of the tonoplast [11], the final fate of the cytoplasmic membrane remaining unknown,

– slow or fast mixing of the vacuolar content with the cytoplasm up to an equilibrium; pH of the cytoplasm (7.2–7.4) [23] driven to pH 5.2–5.4 by the vacuolar medium, resulting in a symplasm at pH 5.2–5.4,

– modification of the concentrations of solutes (electrolytes, neutral substances, proteins) reaching an equilibrium, change in

their coefficients of diffusion, and alteration of osmotic equilibria and viscosities,

– unknown alterations in the apoplasm [pH, solubility of cell wall polysaccharides (pectic substances, cross-linking *via* oxidation (?), permeability of the walls), cell wall-bound proteins (extensin, peroxydase), loosening of the cell walls since heat-treated pods are no longer rigid],

– heat denaturation of proteins: extensive in the case of the β-glucosidase [9, 11, 16, 19, 21], limited for the peroxidase activity [9, 21], unknown for other proteins; precipitation of unknown proteins at their isoelectric pH_i due to alteration of pH of the cytoplasm,

– hydrolysis of the β-glucosides [7–9], being initially stored in the vacuole and put in the presence of the cytoplasmic β-glucosidase by decompartmentation,

– other unknowns.– oxidation of unknown nature (browning, blackening, protein "fining" by tannins?)

Examination of the time curves of hydrolysis of phenolic β-glucosides revealed that all of them were slowly hydrolyzed (*figures 3, 7*): actually, this is one of the aims of the curing process in which the maximum production of vanillin is desirable [2, 7–9, 15, 22]. The final extent of glucovanillin hydrolysis is clearly related to the temperature used for the heat treatment: the higher the temperature, the lower the percent of hydrolysis, *i.e.*, the lower the amount of residual β-glucosidase. We have here a classic phenomenon of heat denaturation of an enzyme, which was well described *in vitro* for a crude vanilla β-glucosidase by Márquez and Waliszewski [12]. The enzymatic nature of the hydrolytic agent is also visible during the first fortnight of storage when reaction velocities (*figure 3*) are zero-order with time, thus with substrate concentration (high during this period), *i.e.*, the enzyme works at its maximum rate V_{max}; they are also proportional to the inverse of temperature treatment, *i.e.*, to the amount of available active enzyme [$v = k(e)$]. Thus, one may state first that, considering the enzymatic hydrolysis of glucovanillin alone, *i.e.*, independently of other enzymatic and/or chemical reactions which may occur, the efficiency of the curing process in terms of maximum attainable amount of vanillin is, independently of the

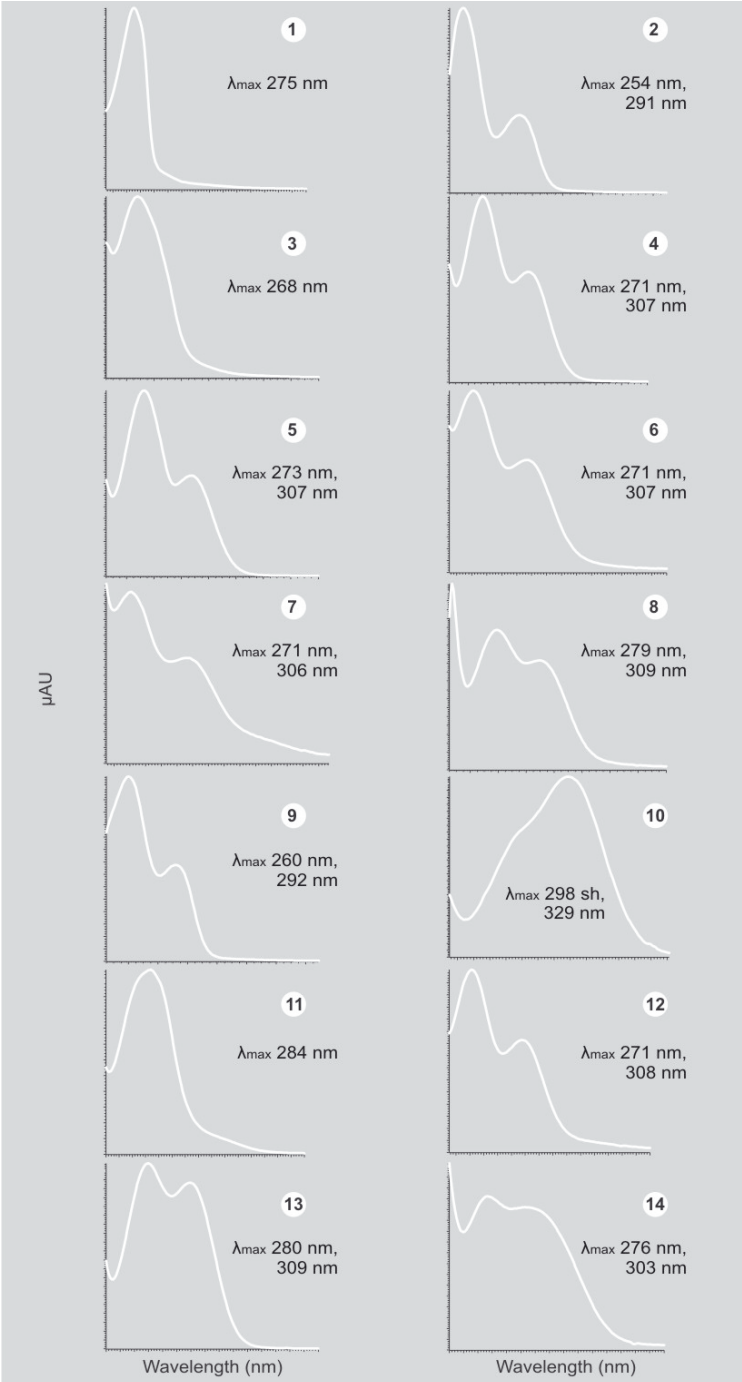

Figure 6.
UV spectra of the compounds detected in the HPLC chromatograms of the extraction media from vanilla pod slices treated at 55 °C. Identities of peaks as follows: 1: unknown glucoside, 2: vanillic acid glucoside, 3: *p*-OHbenzaldehyde glucoside, 4: unknown aglycon, 5: glucovanillin, 6: unknown aglycon, 7: unknown aglycon, 8: unknown aglycon, 9: vanillic acid, 10: a hydroxycinnamic acid derivative, 11: *p*-OHbenzaldehyde, 12: unknown glucoside, 13: vanillin, 14: unknown.

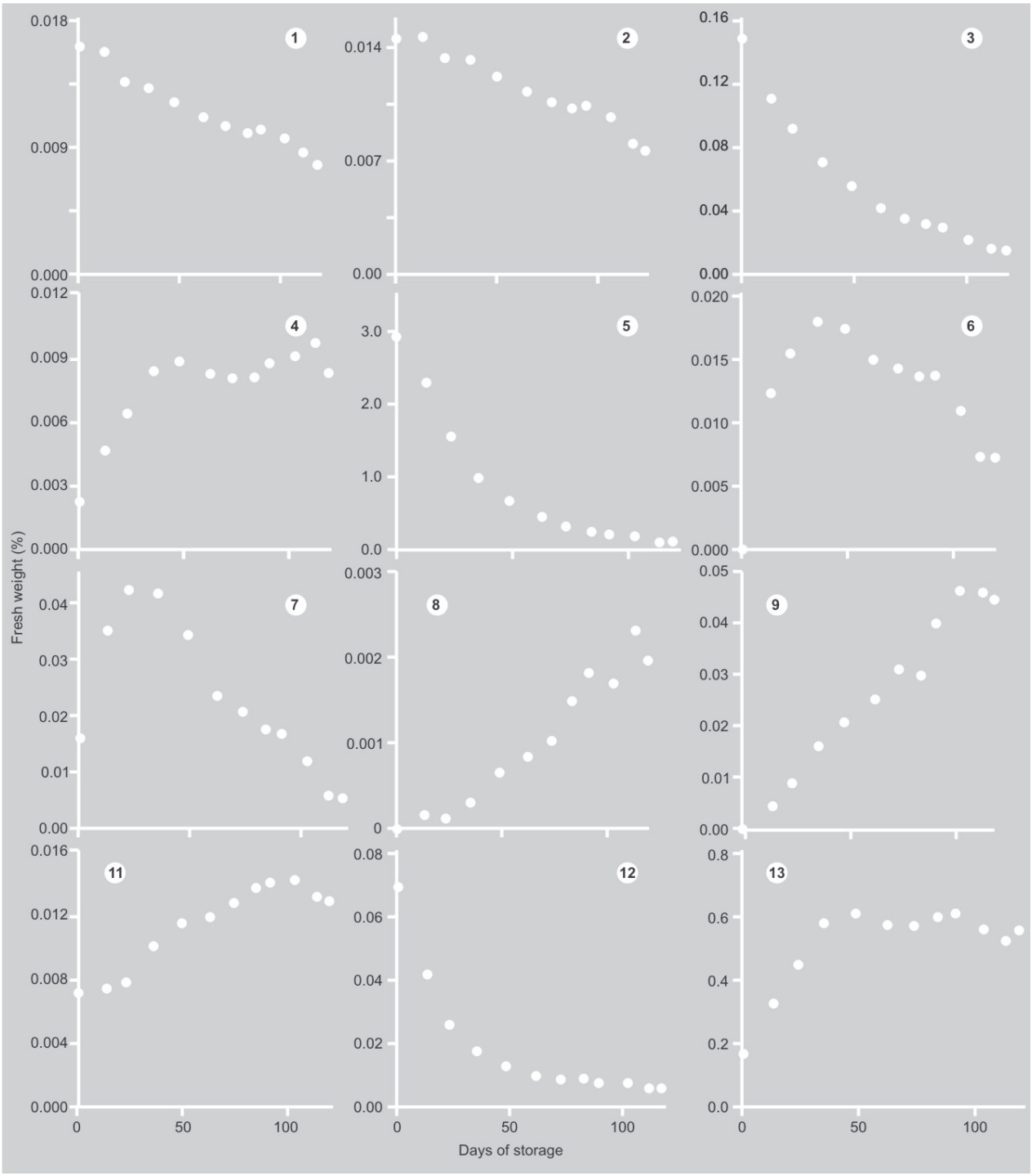

Figure 7.
Evolution of the compounds detected in the HPLC chromatograms of the extraction media from vanilla pod slices treated at 55 °C. Identities of peaks as follows: 1: unknown glucoside, 2: vanillic acid glucoside, 3: *p*-OHbenzaldehyde glucoside, 4: unknown aglycon, 5: glucovanillin, 6: unknown aglycon, 7: unknown aglycon, 8: unknown aglycon, 9: vanillic acid, 11: *p*-OHbenzaldehyde, 12: unknown glucoside, 13: vanillin.

initial glucovanillin content, directly linked to the temperatures involved and the lengths of time when the pods are exposed to heat, and this at constant water content. Since during the first two critical steps of the traditional process ("killing" and sweating), the pod dry matter content changes little [8], the above proposals remain valid in the frame of the vanilla curing.

Thus, optimization of a curing pattern would be a compromise between the speed and extent of heat denaturation and the optimization of the working conditions under which the residual β-glucosidase will work afterwards: for example, a long "killing" treatment at a moderate temperature (*e.g.*, 50 °C, if high enough to arrest the vegetative life of the pod and to disrupt the vacuolar membrane) will allow a maximum rate of hydrolysis of β-glucosides, while enzyme activity will be lost progressively to an unknown residual amount. Márquez and Waliszewski suggested, from their *in vitro* study of thermal inactivation of vanilla β-glucosidase, such a "cold killing" [17]. The sweating step is also important since pods are wrapped in woolen cloth, placed in closed boxes lined with the same cloth, and stored for a while [15], conditions under which the residual heat from the "killing" step is kept, *i.e.*, the temperature remains sufficiently high for a day [8, 15] at a level where the residual β-glucosidase works rapidly before progressively falling by heat denaturation; this step was likely empirically promoted for this reason.

On top of that, the purified vanilla β-glucosidase is very unstable under pH 5.6 (complete loss of activity in 4 h at 20 °C) [5]. Since the vacuole occupies ~93% of the intracellular volume [4], and because upon senescence or curing the tonoplast seems to be disrupted [11], it is reasonable to think that, under these conditions, the vacuolar juice and the cytoplasm (pH ~7.2–7.4, [23]) are mixed, and that the pH measured on a crushed vanilla pod (pH ~5.2) is the vacuolar one. Thus, in addition to the heat denaturation occurring during the curing process, the β-glucosidase is also affected by the acidic pH generated by the decompartmentation [11] and blending of cell contents [24].

In the end, infinitesimal amounts of β-glucosidase are available for glucovanillin hydrolysis, which partly explains the length of the curing process; it is therefore not surprising that authors have not been able to measure this activity in heat-treated pods [9, 25]. However, this impossibility is not contradictory with the fact that all phenolic aglycons initially present as β-glucosides are produced by enzymatic hydrolysis. Kanisawa reported two β-glucosidases in vanilla [19], and this proposal was taken by Dignum *et al.*, who proposed that the glucosidase "specific" to glucovanillin would resist the heat denaturation while the "non-specific" one, also active on *p*-nitrophenyl β-glucoside, would be denatured [9]. We have doubts about the putative existence of this second enzyme since Kanisawa never published a complete purification procedure or an extensive characterization (substrate specificity and kinetics) of this so-called "specific" enzyme; furthermore, we measured for several years the β-glucosidase activities on *p*NPG and glucovanillin and systematically found that their ratio was constant in all instances (diverse purification steps, direct measurements on vanilla pods, etc.).

Kinetics of the pure vanilla β-glucosidase in dilute media shows a typical Michaelis mode of functioning with the reaction rate being first-order for low glucovanillin concentrations ($<< K_m$) and zero-order, *i.e.*, V_{max}, for high levels ($>> K_m$) ([5], this work); the enzyme, a tetramer [5], does not show an allosteric behavior under these dilute conditions (see below). We have no explanation for the reason why formerly published K_m for *p*-nitrophenyl β-glucoside and glucovanillin [5] differ from those given in this work which, in the end, are closer, and even identical for glucovanillin, to those published by Dignum *et al.* [10]. This constant was not affected when pH was changed to 5.2 with the presence of 150 mM glucose.

Kinetics of the β-glucosidase *in vivo*, *i.e.*, in the heat-treated tissues, shows, against glucovanillin, an apparent allosteric behavior, compatible with the enzyme being a tetramer. Fitting the experimental curve [v = f(s)] with a Hill equation yielded the best fit with a Hill coefficient of 2.9, indicating a possible positive cooperativity between the monomers in the ligand binding. The

$K_{0.5}$ for glucovanillin was found at 21.8 mM, *i.e.*, four times higher than the K_m of the pure β-glucosidase in a dilute medium: the β-glucosidase thus exhibits *in vivo* a lower affinity for its substrate than in dilute conditions; however, one should bear in mind that concentrations of solutes, including proteins, in the aqueous phase of the heat-treated tissues might change the affinity constant and they remain unknown at the moment.

Linear modes of hydrolysis of unknown glucoside (n° 1) and vanillic acid glucoside also suggest that the β-glucosidase does not work *in vivo* under a Michaelian mode, since due to their very low concentrations, the reaction rate must vary linearly (first-order with concentration) while it was constant; vanillic acid glucoside was hydrolyzed much more slowly than glucovanillin, as reported by Dignum *et al.* [9, 10].

Thus, this apparent change in its mode of functioning, from Michaelian to allosteric, if actual, requires extensive research, taking into account the physico-chemical status of the symplasmic medium after heat treatment.

5. Conclusion

Very slow kinetics of hydrolysis of phenolic β-glucosides in heat-treated vanilla pods results, after heat denaturation, from the action of residual trace amounts of β-glucosidase. We showed that this β-glucosidase, a tetramer, does not behave in heat-treated vanilla tissues as a Michaelian enzyme, but might work under an allosteric mode with a reduced affinity for its natural substrate, glucovanillin.

Acknowledgements

Thanks are due to Dr. M. Grisoni (CIRAD, Réunion Island) for the gift of vanilla pods.

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Cinética del funcionamiento *in vivo* de una β-glucosidasa hacia la liberación de la glucovainillina, y de glucósidos fenólicos conexos en la vaina de la vainilla sometida a un tratamiento térmico (*Vanilla planifolia*, Orchidaceae).

Resumen — Introducción. El procedimiento tradicional de preparación de las vainas de vainilla engloba una etapa de escaldado y otra de secado, durante las cuales las vainas se someten al calor (35–65 °C) de duración variable. Por mucho que se sepa que la liberación de la vainillina y de otras composiciones fenólicas a partir de sus respectivos glucósidos no aromáticos se debe a la acción de una β-glucosidasa endógena, se desconocen aún sus parámetros cinéticos de funcionamiento *in vivo*. **Material y métodos**. Primero se trataron unas vainas verdes maduras durante 2 h, 50 °C, 55 °C y 60 °C, respectivamente, y luego se almacenaron durante 118 días a 27 °C. Se extrajeron los glucósidos de fenoles así como sus respectivos aglicones en intervalos regulares durante el periodo de almacenamiento y se analizaron mediante HPLC. **Resultados y discusión**. Se hidrolizaron lentamente todos los glucósidos de fenoles durante el periodo de almacenamiento con liberación de vainillina, *p*-hidroxibenzaldehído, ácido vainíllico y otros aglicones desconocidos. Lo esencial de la actividad β-glucosidasa se desnaturalizó debido al calor durante el tratamiento preliminar. El análisis de sus parámetros cinéticos mostró que la encima adopta *in vivo* un modo de funcionamiento alostérico con una afinidad por la glucovainillina más débil que *in vitro*, en donde se comporta como una enzima michaeliana. **Conclusión**. La confirmación del mecanismo alostérico de la β-glucosidasa *in vivo* de la vainilla requiere un estudio profundizado.

Francia / *Vanilla planifolia* / vainilla / procesamiento / calor / glucovanillina / β-glucosidasa / constante de afinidad / alosterismo