Characterization of a Pterostilbene Dehydrodimer Produced by Laccase of *Botrytis cinerea*

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ABSTRACT

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In the interaction between grapevines and *Botrytis cinerea*, one of the main aspects of pathogenicity is fungal ability to degrade phytoalexins synthesized by the plant in response to infection. Laccase-like stilbene oxidase activity in liquid cultures of *B. cinerea* has been shown to be

related to the decrease of phytoalexin concentrations. Recent research and results presented in this paper determined the chemical structure of a pterostilbene metabolite produced by *B. cinerea*. Study of degradation of pterostilbene that has just one free hydroxy phenyl group function allowed us to determine the oxidative dimerization process undergone by grapevine phytoalexins after *B. cinerea* infection. The phytopathological significance of this degradation process in the *B. cinerea* interaction has also been discussed.

Grapevines can synthesize antifungal compounds, phytoalexins (5,9,43), in response to elicitation such as an infection by *Botrytis cinerea*, the causal agent for gray mold (15,17,23,27). Their chemical structure is based on a polyphenol-type stilbene (14,20), resveratrol (*trans*-3,5,4'-trihydroxystilbene) (23-25) (Fig. 1A). In addition to resveratrol, the family of grapevine phytoalexins covers piceid (resveratrol-3-O β -glucopyranose) (46) (Fig. 1B); pterostilbene, a 3,5-dimethylated resveratrol (8,21,22,24) (Fig. 1C); and ϵ -viniferin, a dehydrodimer of resveratrol (21,24,25) (Fig. 1D). Fungitoxicity of these phytoalexin-type compounds (44) has been defined: ϵ -viniferin (21,24) and pterostilbene (22,36) have a high fungitoxicity, though resveratrol, the major compound of the grapevine phytoalexin response, has the lowest activity (1,13,21). The phytoalexin response can, therefore, be qualified either by quality parameters with, for example, the response rapidity and the type of synthesized compounds or by quantity with the resveratrol concentration.

In the interaction between grapevines and *B. cinerea*, it is necessary to study the fungal pathogenicity, i.e., its ability to alter the natural resistance put in place by the plant (15,30,41,42,44). Indeed, this fungus has an enzymatic pool able, for example, to lyse cell walls (38,45) or to oxidize polyphenols with an extracellular enzyme, a laccase-like stilbene oxidase (2,6,29,33). Consequently, direct evidence has been obtained that resveratrol and pterostilbene could undergo degradation by this extracellular enzyme produced by *B. cinerea* (12). Since the first work of Hoos and Blaich (12), much attention has been paid to the biological significance of the laccase-mediated degradation of stilbene phytoalexins, especially in relation to the pathogenicity of *B. cinerea* to grapevines (18,37,40). Recently, we have shown (4) that resveratrol me-

tabolism by laccase includes an oxidative dimerization process leading to a resveratrol dehydrodimer (Fig. 1E), analogous to ϵ -viniferin produced by grapevines (25,34). Studies, in the same conditions, on degradation of pterostilbene that has one free hydroxy phenyl group became necessary to propose for these stilbenes their site of degradation by *B. cinerea* laccase. Characterization of one pterostilbene metabolite by spectral results and data obtained from nuclear magnetic resonance (NMR) analyses allowed us to understand more about grapevine phytoalexin metabolism by *B. cinerea* and its relation with fungal pathogenicity.

MATERIALS AND METHODS

Chemicals. *trans*-Pterostilbene was obtained from R. Pezet and V. Pont (Swiss Federal Agricultural Station of Changins) (35).

Fungal isolates and culture conditions. The strain of *B. cinerea* used in this study (RFA2) was isolated from grapes and obtained from P. Leroux (INRA of Versailles, France).

The strain was cultured on potato dextrose agar (Bio Merieux, Lyon, France) in petri dishes and maintained at 21°C with a 12-h light photoperiod. Culture filtrates of *B. cinerea* (37) were obtained by growing the fungus in the medium created by Fournioux and Bessis (7). The medium was distributed into 2-liter Erlenmeyer flasks (800 ml per flask) and adjusted to pH 5.2 before inoculation with a conidia suspension (containing about 2×10^6 conidia) obtained as previously described (40). Liquid cultures of *B. cinerea* were incubated for 15 days at 21°C on a reciprocal shaker (100 strokes per h) with a 12-h light photoperiod.

Extraction of proteins from culture filtrates of *B. cinerea*. After 15 days of culture, the filtrates were concentrated in vacuo (35°C), the initial volumes of the filtrates being reduced by 80%. Acetone kept at -20°C was added to the concentrated filtrates in a proportion of 3:2 (vol/vol). A precipitation of extracellular proteins was realized after 1 h at 4°C. Centrifugation at $10,000 \times g$ for 20 min allowed us to obtain a protein pellet that was redissolved in phosphate-citrate buffer (0.1 M, pH 3.4) in a proportion of 3 ml of buffer per 2-ml equivalent of concentrated filtrates.

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After centrifugation at $10,000 \times g$ for 10 min, the supernatant containing the protein fraction was kept at -20°C before use.

Incubation of pterostilbene in the presence of culture filtrate proteins of *B. cinerea* and extraction of the pterostilbene metabolite. An ethanolic solution (2 ml) of pterostilbene (1 mg/ml) was added to 20 ml of the phosphate-citrate buffer containing extracellular proteins of *B. cinerea* ($3.2 \mu\text{g/ml}$) (3). We verified, using a spectrophotometric test as described by Sbaghi et al. (40), that the laccase-like stilbene oxidase activity was present in purified protein extracts.

Reaction was stopped after 2 h by extraction using ethyl acetate in a proportion of 1:1 (vol/vol). Organic phases were concentrated under vacuum at 35°C . The dried extract was then redissolved in absolute ethanol before being analyzed.

Characterization of the pterostilbene metabolite by high performance liquid chromatography (HPLC) analysis coupling photodiode array detection and fluorometry. Analyses using the HPLC method described by Jeandet et al. (16) to identify and quantify grapevine phytoalexins (*Vitis* spp.) were performed to show pterostilbene degradation after successive times of incubation with protein extracts (0, 30, and 60 min) and to follow occurrence of its metabolite.

Thin layer chromatography (TLC) semipreparative purification of the pterostilbene metabolite. The pterostilbene metabolite was purified by semipreparative TLC on reversed-phase plates (RP18 F254S; E. Merck AG, Darmstadt, Germany) in methanol/water (7:3, vol/vol). After 3 h of elution, revelation was obtained under long wavelength UV light (366 nm). A pterostilbene standard allowed us to identify the nondegraded pterostilbene.

^1H NMR analyses and ^1H - ^1H (correlation spectroscopy, COSY) correlations. ^1H (500 MHz) spectra and ^1H - ^1H (500 MHz) (COSY) correlations were recorded on a Brüker DRX 500 spectrophotometer (Brüker S.A., Wissembourg, France). ^1H and ^1H - ^1H NMR chemical shifts (δ) were reported in parts per million (ppm) relative to SiMe_4 as an external reference.

RESULTS AND DISCUSSION

HPLC analysis coupling photodiode array detection and fluorometry, a useful method for stilbene identification (16), showed that the decrease of pterostilbene concentration was correlated to the appearance and then the rise of a major fluorescent compound (retention time = 24.5 min). The UV spectrum of this compound from 200 to 400 nm showed two bands of absorption maxima, between 315 to 330 nm and 290 to 310 nm, characteristic of *trans*-stilbenes (10,25). These spectral data suggested that this metabolite has kept, at least in part, a *trans*-stilbenic structure analogous to the compound resulting from resveratrol oxidation by the laccase-like stilbene oxidase of *B. cinerea* (4,26).

Purification of this metabolite for a full characterization was performed by semipreparative TLC on reversed-phased C18; observation under UV light disclosed for this product a blue fluorescence characteristic of *trans*-stilbenes.

A first analysis by ^1H NMR suggested that this compound was a mixture of two isomers: the *trans*- and the *cis*-ethylenic isomers. For the *trans*-ethylenic system, two doublets at the chemical shift (δ) 7.0 (spin-spin coupling constant [J] = 16.5 Hz) and 7.22 ppm (J = 16.5 Hz)

Fig. 1. Chemical structures of the grapevine phytoalexin-type compounds A, *trans*-resveratrol; B, piceid; C, *trans*-pterostilbene; D, ϵ -viniferin; and E, resveratrol *trans* dehydrodimer.

were observed. The characteristic signals for a *cis*-ethylenic bond were obtained at δ 6.56 ($J = 12.2$ Hz) and 6.44 ppm ($J = 12.2$ Hz). As it is well established that a stilbene can undergo photochemical isomerization (10,16), the pterostilbene metabolite was exposed to UV radiations (366 nm) for 30 min. A second ^1H NMR analysis then showed that the intensity of signals at δ 7.00 and 7.22 ppm was decreased, whereas the intensity of signals at δ 6.56 and 6.44 ppm increased after UV exposure. This compound has thus undergone a photochemical isomerization that is a typical reaction for *trans*-stilbenes (10,16). *trans*-Pterostilbene degradation lead to a major compound that was in the *trans*-ethylenic form; but its revelation under a 366-nm wavelength used for TLC semipreparative purification resulted in isolation of its two isomeric forms. Characterization of the pterostilbene metabolite was thus achieved with its *cis*-ethylenic isomer.

Data obtained with the resveratrol metabolite produced by *B. cinerea* laccase under the same conditions (4) and results obtained from high resolution electronic impact mass spectrometry (EIMS) examination (70 eV, M^+ calculated 510.20424, M^+ found 510.20609, molecular formula $\text{C}_{30}\text{H}_{32}\text{O}_6$) suggested a dehydrodimeric structure for the pterostilbene metabolite, the *cis* structure presented in Figure 2A (Fig. 2A, protons [H] and carbon [C] numbers).

To verify this structure (Fig. 2A), detailed comparisons between ^1H NMR analysis before and after isomerization and COSY cor-

relations (^1H - ^1H) were performed. ^1H NMR data at δ 8.45 ppm showed only one phenolic function. Two groups of two equivalent methoxy functions were determined by ^1H NMR analysis at δ 3.75 (singlet, 6H) and 3.65 ppm (singlet, 6H). Protons at δ 6.30 ($J = 1.84$ and 2.44 Hz; H-4E) and 6.42 ppm ($J = 2.44$ Hz; H-4B), whose signals were triplets, showed COSY correlations with protons at δ 6.40 ($J = 1.83$ Hz; H-2E and H-6E) and 6.36 ppm ($J = 2.44$ Hz; H-2B and H-6B), respectively, whose signals were doublets. Ring (Fig. 2A, rings B and E) structure was defined. A proton at δ 7.18 ppm ($J = 1.83$ and 8.54 Hz; doublet of doublets, H-6D) was correlated with a proton at δ 6.82 ppm ($J = 7.94$ Hz; doublet, H-5D) and with a proton at δ 6.96 ppm (broad singlet, H-2D). These results permitted us to confirm ring (Fig. 2A, ring D) structure. Assignments of ring (Fig. 2A, ring A) protons were determined by characteristic chemical shift (δ) of aromatic protons and their COSY correlations. Two signals corresponding to nonaromatic protons at δ 4.48 ($J = 7.93$ Hz; H-2C) and 5.58 ppm ($J = 7.93$ Hz; H-1C) showing COSY correlations were assigned to ring (Fig. 2A, ring C) protons.

Data obtained by Hölscher and Schneider (11), Reinerio et al. (39), and Breuil et al. (4) added to these results and allowed us to confirm the structure presented in Figure 2A. Proton assignments obtained by NMR analysis are summarized in Table 1.

The *trans*-metabolite structure was determined by analogy with the *cis* structure of the pterostilbene metabolite (Fig. 2B).

Degradation of grapevine stilbenes by laccase-like stilbene oxidase of *B. cinerea* thus ended in synthesis of a major compound with a dehydrodimeric structure.

The structure of the pterostilbene metabolite showed that grapevine phytoalexins undergo oxidative dimerization during their metabolism by *B. cinerea* involving the 4'-hydroxyl of the stilbene skeleton. It is well known that laccase, which is a *p*-*o*-diphenol oxidase (oxygen oxido-reductase, EC 1.10.3.2) synthesized either by plants (19,31,32) or by fungi (6,29,33) provides oxidation of phenol functions situated in a *p*-*o*-position to give quinonic structures (31,32). We suggest that dimerization of grapevine phytoalexins should have as an intermediate compound a quinone that would lead to a radical cation with a substituted *p*-hydroxy function explaining chemical reactivity of stilbene phytoalexins according to the results obtained by Majima et al. (28). Thus the 4'-hydroxy phenyl group may control metabolism of grapevine phytoalexins by laccase of *B. cinerea*.

From now on, it will be interesting to discuss the biological significance of the laccase-mediated metabolism of phytoalexins in the *B. cinerea*-grapevine interaction.

It has already been reported (40) that pathogenicity of *B. cinerea* isolates to grapevines is strongly associated with their ability to degrade stilbene phytoalexins, though nonpathogenicity seems to be related with an inability to do so. These observations suggest that phytoalexin metabolism by laccase of *B. cinerea* may correspond to a process by which the fungus can escape from the action of the phytoalexin.

Generally, hydroxystilbenes such as resveratrol and their methylated derivatives are scarcely soluble in water. Study of their biological activity requires solubilization through the addition of ethanol at a final concentration of 4% (1,36). Monomeric stilbenes are relatively more soluble in water than the corresponding dimers.

Fig. 2. A, *cis* and B, *trans* chemical structures of the pterostilbene metabolite produced by laccase of *Botrytis cinerea*.

TABLE 1. Proton assignment determination of the *cis* structure of the pterostilbene metabolite^x

	Protons															
	2C	1C	4E	2B6B	4B	2E6E	H _a	H _b	5D	3A5A	2D	6D	2A6A	OH(4A)	OCH ₃	OCH ₃
d (ppm)	4.48	5.58	6.30	6.36	6.42	6.40	6.44	6.56	6.82	6.86	6.96	7.18	7.23	8.45	3.71	3.65
<i>J</i> (Hz) ^y	7.93	7.93	1.84–2.44	2.44	2.44	1.83	12.20	12.20	7.94	8.55		1.83–8.54	8.55			
Signal ^z	d	d	t	d	t	d	d	d	d	d	s	dd	d	s	s	s

^x Nuclear magnetic resonance data were obtained with analyses of the ^1H (500 MHz) spectra and the ^1H - ^1H (500 MHz) (correlation spectroscopy [COSY]) correlations.

^y *J* = spin-spin coupling constant.

^z s = singlet, d = doublet, t = triplet, and dd = doublet of doublets.

For example, pterostilbene is sparingly soluble in water (ca. 5 µg/ml), though its condensation product (the dehydrodimer identified in this work) is thoroughly insoluble in water (<1 µg/ml). These values are considerably lower than the concentrations of stilbene phytoalexins required to obtain fungitoxic activity against *B. cinerea* (i.e., ID50 concentrations are 20 and 90 µg/ml for pterostilbene and resveratrol, respectively) (1,36).

Due to this low solubility, it was impossible to determine the biological activity of the pterostilbene dehydrodimer against *B. cinerea*, even when using the practical test described by Adrian et al. (1). In the case of pterostilbene, oxidative dimerization by laccase of *B. cinerea* results in a stilbene of a high molecular weight (twice that of the monomeric form) with four methoxy groups. Thus, metabolism of phytoalexins by *B. cinerea* may appear as an insolubilization of the products formed. The fungus could, therefore, escape from the action of phytoalexins by transformation of these compounds to other products with increasing molecular weight (32) and hydrophobicity. Moreover, in the case of resveratrol, Dercks and Creasy (5) stated that ε-viniferin, a dimeric form of resveratrol analogous to the dimer produced by *B. cinerea* (4), is less stable (soluble) in water than resveratrol itself, decreasing its biological activity against *B. cinerea*.

These observations reinforce the involvement of the laccase-mediated oxidation of stilbenes in the *B. cinerea*-grapevine interaction.

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