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Degradation of procyanidins by *Aspergillus fumigatus*: Identification of a novel aromatic ring cleavage product

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Abstract

Aspergillus fumigatus was able to grow on apple-purified procyanidins (PCs). PCs concentration decreased 30% over the first 60 h of liquid fermentation. The mean degree of polymerization (DPn) of apple-purified PCs increased from 8 to 15 during the fermentation. A fungal enzyme extract from the liquid fermentation was used to study procyanidin B2 [(−)-epicatechin-(4β-8)-(−)-epicatechin] degradation. The major degradation product (PB2-X) had a retention time of 10.5 min and a molecular mass at *m/z* 609. High-performance liquid chromatography/multiple fragment mass spectrometry (HPLC/MS^n) was used for the structural characterization of PB2-X as well as that of thiolysis-treated PB2-X. Twelve fragment ions at *m/z* 565, 547, 457, 439 (two fragment ions), 421, 413, 377, 395, 351, 287 and 277 were completely identified. It was therefore deduced that the terminal unit of procyanidin B2 dimer was modified by an oxygenase from *A. fumigatus* leaving the extension unit intact. In addition, FT-IR analysis confirmed a lactone formation in (−)-epicatechin moiety involved in oxidative degradation. Two reaction schemes were postulated for the interpretation of the results.
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1. Introduction

Procyanidins (PCs) or condensed tannins are phenolic compounds widely distributed in the plant kingdom. Commonly known as potent anti-oxidants, PCs are present in a wide variety of fresh fruit and processed beverages [1]. Recent reports have suggested that dietary consumption of PCs is associated with health benefits through, for example, oxygen free radical scavenging activity [2,3]. PCs have other properties including their ability to bind strongly to proteins, reducing significantly nutritional value of feed when used in animal diets at high concentrations [4,5]. PCs are considered to be primarily responsible for the astringent properties of cider beverages [6]. In addition, PCs inhibit several enzymatic reactions [7,8]. In order to increase the nutritional value of PC-containing products, several attempts have been carried out to degrade these anti-nutritional compounds through enzyme catalysis [9,10]. It has also been proposed that PCs involved in plant metabolism play an important role in defense against microorganisms [4]. Although PCs have been shown to be particularly resistant to degradation and to have toxic effects, some microorganisms, mainly bacteria, can tolerate and degrade condensed tannins [11]. However, the information on PCs degradation by fungi remains poor mainly due to a lack of available purified substrates. Moreover, enzymatic mechanisms have not been established as standards are scarce and expensive. Some fungi

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have been studied for their ability to degrade monomeric aromatic compounds [12] such as protocatechuic acid [13] or catechin [14,15]. In 1994 Nguz et al. [16] reported for the first time the degradation of PCs by *Penicillium.* Later, in 1996 Gamble et al. [17] used solid-state ^{13}C NMR technique to compare two strains of white rot fungi (*Ceriporiopsis subvermispora* and *Cyathus stercoreus*) for their ability to catabolize condensed tannins. Recently, using the same technique, Vane et al. [18] observed that tannins of wood decayed in the presence of an ascomycete fungus (*Hypocrea sulphurea*). However, these reports were limited to the simple observation of the degradation events and, to date, no information is available on the products obtained as well as the enzymes involved in the degradation pathways.

Natural PCs are oligomers and polymers of flavan-3-ol units such as (+)-catechin or (−)-epicatechin [19] mostly linked by C4−C8 or C4−C6 interflavan bond (Fig. 1). Each plant, source of PCs, contains a different distribution of these polymers. Procyanidins are therefore characterized by their average degree of polymerization (DPn) that corresponds to the average number of flavanol units making up the polymer. The DPn is measured as the molar ratio between all units (terminal and extension) over the terminal units. The ratio obtained after procyanidin depolymerization in the presence of benzylthioether (thiolysis) can be quantified by HPLC analysis of the thiolysis medium [20,21]. In addition, the use of the thiolysis-based technique has allowed the characterization [22] and quantification [23,24] of the constitutive units of procyanidins. The method has been applied to a large variety of fruit [25] including apple [26,27], grape [28], pear [29] and coffee pulp [30].

In recent years, mass spectrometry (MS) has been increasingly applied to the structure determination of natural compounds due to sensitivity, rapidity and low concentration of samples. In addition, the coupling of liquid chromatography/mass spectrometry (LC−MS) with electrospray ionization (ESI) techniques has proven to be a powerful tool for the identification of PC structures in foods [31,32]. Following identification, PC structures can be elucidated through the study of fragment ions obtained by multiple fragment mass spectrometry (MS^n).

The aim of the present study was to demonstrate that specific fungi were able to metabolize apple-purified procyanidin oligomers. In doing so, *Aspergillus fumigatus* produced extracellular enzymes that were then used as *in vitro* tools to study the first step in the degradation pathway of a model procyanidin dimer, namely procyanidin B2 [(−)-epicatechin-(4β-8)-(−)-epicatechin].

2. Materials and methods

2.1. Chemicals

(+)-Catechin and (−)-epicatechin were obtained from Sigma Chemicals, France. Acetonitrile (HPLC grade) and

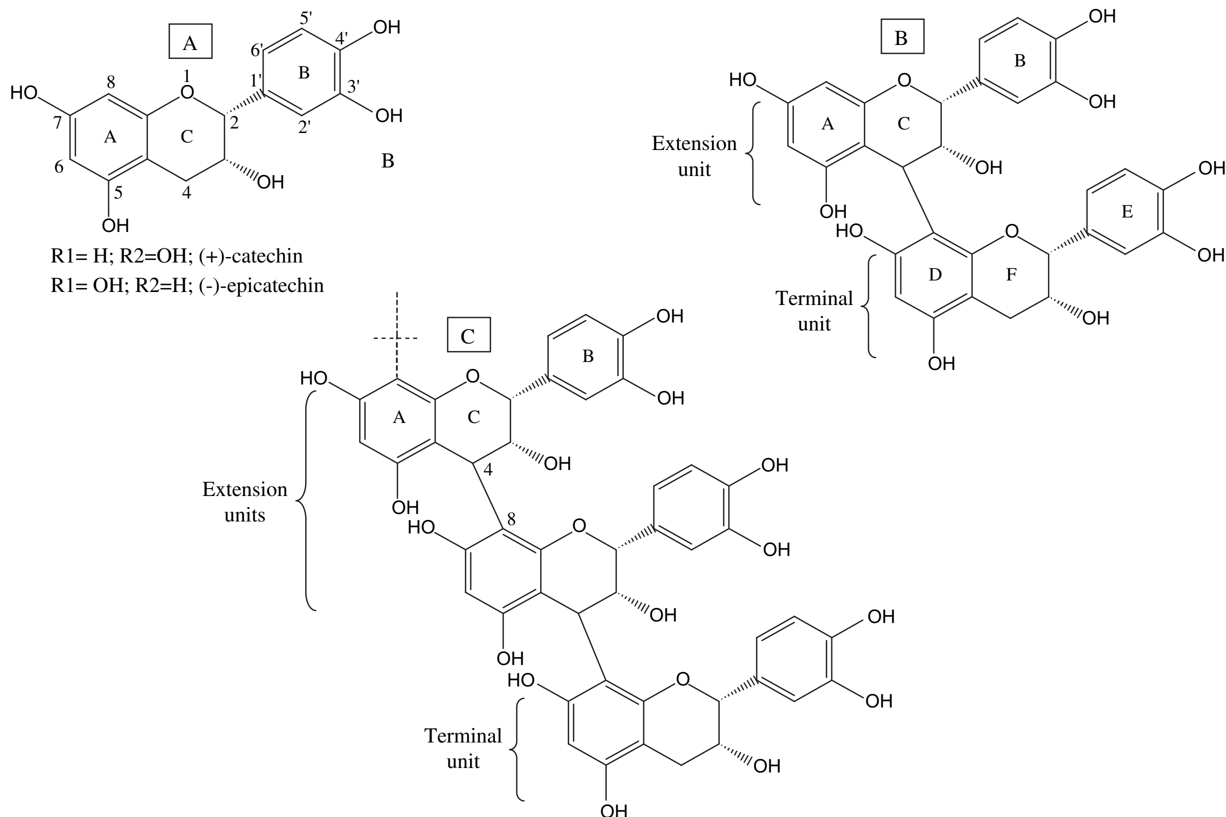


Fig. 1. General structures of flavan-3-ol units: (A) (+)-catechin and (−)-epicatechin monomers, (B) procyanidin B2 dimer (PB2), and (C) procyanidin oligomers with C4−C8 linkage.

acetic acid were from Biosolve (France) and benzylmercaptan was from Merck (Germany). Procyanidin B2 dimer [(−)-epicatechin-(4β-8)-(−)-epicatechin] was purchased from Extrasynthèse (Lyon, France). (−)-Epicatechin-benzylthioether standard was provided by N. Marnet (URC-BFL, INRA, Le Rheu, France).

2.2. Purification of polymerized procyanidins

Freeze-dried apple powder of a cider variety (cv. *Marie Ménard*) was successively treated with hexane, methanol and aqueous acetone as previously described [27]. The aqueous acetone fraction was evaporated and freeze-dried as previously reported by Guyot et al. [26]. The freeze-dried powder (4 g) was suspended in 40 ml of 2.5% acetic acid in water (v/v). The suspension was filtered through a Millipore filter (0.45 μm) and injected into a preparative HPLC system (binary pump system Dynamax SD 300 Rainin, UV−visible detector Dynamax UV1 Rainin and the EZChrom System) set at 280 nm. The column was a Lichrospher 100 RP C18, 12 μm particle size, 50 × 200 mm length (Merck, Germany). The flow rate was 30 ml min^{-1} and the conditions were: solvent A (CH_3COOH 2.5%, H_2O, v/v) and solvent B (CH_3CN); the gradient applied was 0−60 min; 100% A isocratic; 60−70 min, 50% A. The fraction rich in PCs was collected, followed by washing and reconditioning of the column to initial conditions. Solvent in the sample was evaporated under vacuum at 30 °C to remove organic solvents. The sample was then freeze-dried.

2.3. Micro-organisms

A. fumigatus was isolated from coffee pulp [33] and grown on Potato Dextrose Agar (Sigma) at 30 °C for 6 days for spore production. Spores were then harvested with 30 ml of sterilized distilled water containing 0.01% (v/v) Tween 80 under agitation provided by a magnetic stir bar. The suspension obtained was used as inoculum of culture medium.

2.4. Culture medium

Culture medium used was essentially as described by Roussos [34], containing (in g l^{-1}): $SO_4(NH_4)_2$, 1.0; KH_2PO_4, 1.3; Na_2HPO_4, 0.12; $MgSO_4$, 0.3; Urea, 0.3; glucose, 2; and 1 ml of oligo-element solution ($FeSO_4 \cdot 7H_2O$, 5; $MnSO_4 \cdot H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; $CaCl_2$, 2). The pH was adjusted to 6 with NaOH (3 M) and the medium was sterilized at 120 °C, 20 min. PCs were dissolved in water, pH was adjusted to 6 and the medium was sterilized by filtration on a Millipore filter (0.22 μm, PVDF). PCs were added aseptically to the culture medium in order to reach a final concentration of 2 g l^{-1}.

2.5. Culture conditions

Experiments were carried out in Erlenmeyer flasks (150 ml) with 15 ml of culture medium inoculated with 5×10^7 spores per gram carbon source. Flasks were incubated at 30 °C with an agitation rate of 120 rpm. All experiments were conducted in duplicate. Un-inoculated medium was used as a positive control to determine whether polyphenols added underwent oxidation during the experiment.

2.6. Biomass estimation

Fungal biomass was measured gravimetrically. Culture medium was filtered through a Millipore filter (0.45 μm, PVDF). The mycelium retained was washed with a saline solution (NaCl, 0.9%) and samples were dried to constancy at 60 °C for 48 h. Biomass concentration was reported as grams of dry weight per liter of culture medium (g l^{-1}).

2.7. Glucose determination

Prior to glucose determination, excess PCs were retained on a C18 cartridge (Waters Co. U.S.A.). The cartridge (1 g) was conditioned with 15 ml of MeOH and equilibrated with H_2O (15 ml). Samples of 5 ml each were passed through the cartridge and after discarding the first 2 ml, 1 ml was collected. Glucose was measured as described by Dubois et al. [35] at 490 nm and compared with a glucose standard curve.

2.8. Extracellular extract

A. fumigatus culture medium was filtered using a Millipore membrane (0.45 μm, PVDF) to retain biomass. Then, as described above, excess PCs from culture filtrate were retained utilizing a C18 cartridge (Waters Co. U.S.A.) of 1 g previously conditioned with MeOH and H_2O. Flow-through obtained was collected, stored at 4 °C and tested for extracellular enzyme activity.

2.9. Enzyme assay

Enzyme activity was determined by HPLC by following the disappearance of PC dimer procyanidin B2 (PB2) at 280 nm. The reaction was carried out at 30 °C for 1 h. Assay mixture contained 200 μl of PB2 dimer solution (1 mM) in phosphate buffer, pH 6 (0.2 M) and 50 μl of extracellular enzyme extract. One unit of activity was defined as the amount of enzyme which catalysed the disappearance of 1 μmol min^{-1}(U) and ml^{-1} (U ml^{-1}) of PB2 at 30 °C.

2.10. Thiolysis−reversed phase HPLC conditions

Thiolysis was carried out according to the procedure described by Guyot et al. [27]. In brief 2 ml of anhydrous MeOH were added to freeze-dried culture medium fermentation samples (4 mg). The reaction was carried out in glass tubes (250 μl) as follows: 50 μl of sample were mixed with 100 μl of benzylthioether solution (5% in anhydrous MeOH) and 50 μl HCl (0.4 N) solution. The mixture was incubated in a water bath at 40 °C for 30 min. For the thiolysis reaction on both PB2 and enzyme-treated PB2 (PB2-X), samples were diluted in anhydrous MeOH and thiolysis was carried out as

described above. The reaction was stopped by transferring samples to an ice bath. HPLC analyses of these samples were performed on a Waters HPLC system with a 717 plus autosampler equipped with a cooling chamber for samples, a 600E multisolvent pump and a 996 photodiode array detector. Compounds were separated on an end-capped Purospher RP-18 column (250 mm × 4 mm, Merck, Germany) at 30 °C with a flow rate of 1 ml min^{-1}, by a gradient elution system (solvent A: aqueous acetic acid, 2.5%, v/v and solvent B: acetonitrile. Initial, 3% B; 0–5 min, 9% B linear, 5–15 min, 16% B linear and 15–45 min, 50% B linear, followed by washing and reconditioning of the column).

2.11. Total procyanidin determination

Total PCs were estimated as reported by Porter et al. [36]. After biomass separation from *A. fumigatus* cultures, supernatant containing PCs was mixed with reactive butanol–HCl. The reaction mixture containing 7 ml of butanol–HCl was introduced into a glass tube (15 ml), and 500 μl PCs culture media were added. Samples were heated at 95 °C for 1 h then cooled at room temperature for 15 min. Color formation was monitored with a spectrophotometer set at 550 nm. PCs were quantified by comparison to a PC standard curve.

2.12. Purification of PB2 and PB2-X (the degradation product of procyanidin B2) for analytical purposes

Purification of PB2 (Fig. 1B) and PB2-X was performed by HPLC. The running and elution conditions were identical to those described above. However, separation was carried out on a 250 × 4.6 mm Atlantis dC18 column (Waters Co. U.S.A.) with a particle size of 5 μm at room temperature. Peaks corresponding to residual PB2 and to PB2-X were collected. Excess acetonitrile and acetic acid were eliminated by evaporation at 30 °C and the residues were freeze-dried. A solution of purified PB2-X was prepared in MeOH and filtered through a 0.45 μm Teflon membrane (PTFE, Millipore) and analyzed by Reversed-phase High-Performance Liquid Chromatography/Electrospray Mass Spectrometry (RP-HPLC–ESI-MS). In addition, PB2-X was also subjected to thiolysis and then analyzed by RP-HPLC–ESI-MS.

2.13. HPLC-ESI-MS analysis

The MS apparatus was an LCQ Deca ion trap (Thermofinnigan, San Jose, CA, U.S.A.) equipped with an electrospray ionization source. All MS experiments were carried out in the negative mode $[M-H]^{-1}$. Nitrogen was used as nebulizing gas. For LC-MS, the source parameters were: spray voltage, 3.7 kV; sheath gas, 65 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature 250 °C. Helium was used as dampening gas. Auto Gain Control mode was used to optimize injection time. ESI-MS was coupled to an HPLC system: HP quaternary gradient Pump 1100 series (Agilent Technologies), autosampler and data were processed by Xcalibur® version 1.2 software. Samples were separated on a Zorbax Eclipse XDB-C18 column (2.1 mm × 150 mm, 3.5 μm, Agilent Technologies). A binary gradient was applied (eluent A, 0.1% formic acid in water and eluent B, 0.1% formic acid in acetonitrile): initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; 15–45 min, 50% B linear, at a flow rate of 0.2 ml min^{-1}. Full scan signals were recorded within the *m*/*z* range of 200–1000.

For infusion analyses, the source parameters were as follows: spray voltage 4.5 kV, sheath gas, 40 arbitrary units; capillary temperature 180 °C. Helium was used as dampening gas for MS^n experiments.

2.14. Infrared spectroscopy

Infrared spectra were performed on a Fourier Transform Infrared (FT-IR) Nexus (Thermo Electron) involving Attenuated Total Reflection (ATR). The instrumental had a spectral resolution of 4 cm^{-1}, and a spectral field of 4000–450 cm^{-1}.

2.15. Oxygen consumption

Oxygen consumption was determined at 30 °C utilizing a Clarke oxygen electrode to monitor oxygen consumption rate. A solution containing 1.6 ml de PB2 dimer (1 mM), in phosphate buffer (0.2 M, pH 6) was introduced into the electrode chamber. Temperature was maintained at 30 °C. The reaction began when the extracellular enzymatic extract (400 μl), free of PCs, was added to the chamber. The amount of oxygen consumed was calculated using the saturation level of dissolved oxygen in water at 30 °C.

3. Results and discussion

3.1. Characteristics of A. fumigatus growth on procyanidins

In an attempt to analyze the degradation pathway of procyanidins by filamentous fungi, a source of polymerized PCs from cider apple (*M. Ménard* variety) was purified. The average degree of polymerization of the PCs fraction was 8 (data not shown). This fraction was used as a carbon source for *A. fumigatus* growth.

PCs concentration decreased 30%, essentially over the first 60 h of fermentation (Fig. 2A). This coincided with biomass production, which peaked around 50 h (Fig. 2B). *A. fumigatus* grew in the absence of glucose (data not shown) but the latter was nevertheless added to the culture medium as biomass production was increased considerably in its presence. Such effects have been documented; for example, Nguz et al. [16] showed that *Penicillium* sp. was able to degrade condensed tannins from sorghum more efficiently when glucose was present in the culture medium. In the present study, glucose was depleted within 72 h of fermentation (Fig. 2B). PCs were not further degraded after 60 h (Fig. 2A). This result may be related to nutrient limitation, indicating that PCs biodegradation could be associated with growth. Similar phenomena have been observed [37]. *A. fumigatus* strain grew in the

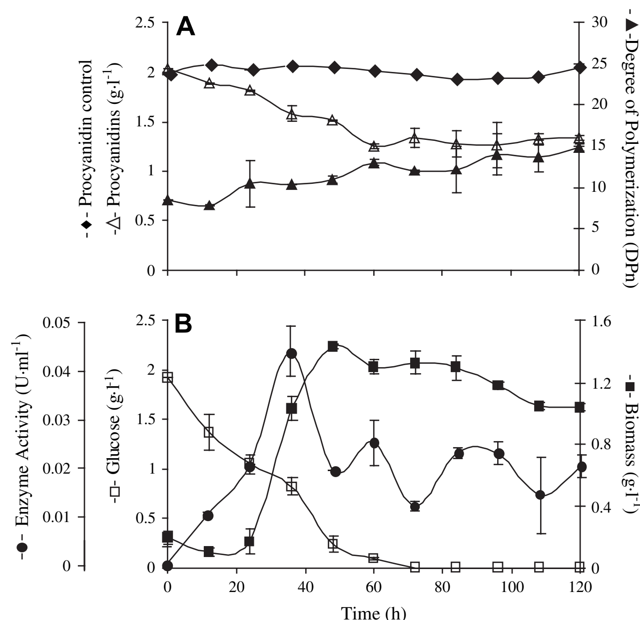


Fig. 2. (A) Variation of total procyanidin concentration over time (△) and average degree of polymerization (DPn) of procyanidins (▲), and un-inoculated control medium (◆), (B) PB2-degrading enzyme activity present during *A. fumigatus* growth in liquid culture (●), growth of *A. fumigatus* on procyanidins-containing medium (■), and glucose consumption (□).

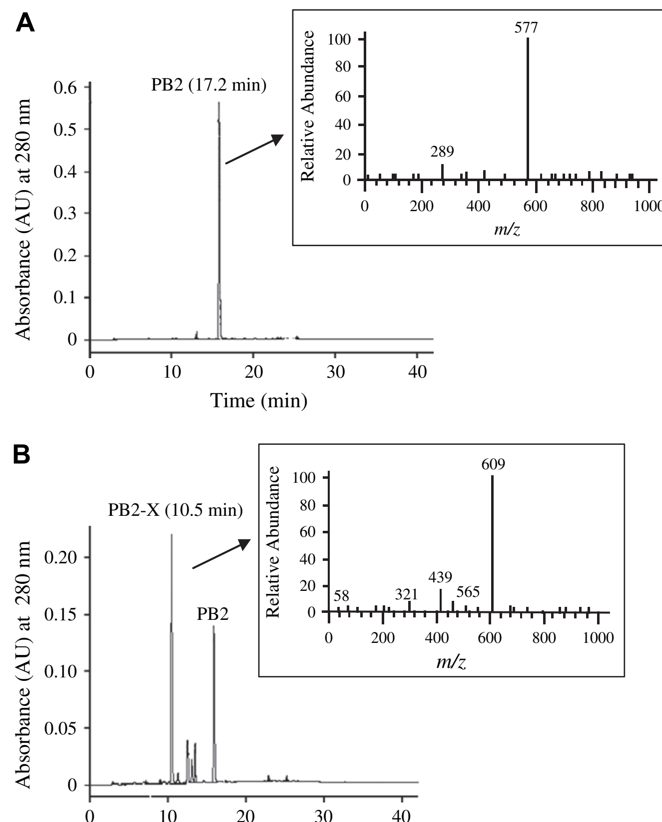


Fig. 3. HPLC elution profile of procyanidins (detection at 280 nm). (A) Elution of PB2 and mass spectrum (boxed) of the purified peak. (B) Elution profile of PB2 and its degradation product (PB2-X) after treatment with enzyme-containing extracellular medium from *A. fumigatus* at 36 h of growth. Mass spectrum of the major purified peak is boxed.

presence of $2\,g\,l^{-1}$ of apple PCs. Inhibition of microbial growth in the presence of tannins has been described [11]. Gonzalez de Colmenares et al. [38] observed that PC extracts from coffee pulp and coffee leaves at a concentration of $2\,g\,l^{-1}$ were effective inhibitors of spore germination of the coffee rust fungus (*Hemileia vastratix*). In the present study, it is to be noted that decreasing concentrations of PCs are correlated with an increase in DPn from 8 at the beginning of the fermentation to 15 at the end of the fermentation (Fig. 2A). PC oligomers (vs. polymers) were preferentially consumed as they disappeared during fungal growth (data not shown). These findings may be correlated with the fact that enzymatic attacks by fungi on higher molecular weight PCs are more difficult. [39]; another chemical explanation could be that the increase of the measured DPn resulted in the alteration of some extension or terminal units which were therefore not considered for quantification after thiolysis degradation. In order to better understand the enzymatic process involved, the degradation mechanism was further investigated using commercially available PB2 as substrate and the fungal culture filtrate as enzyme source.

3.2. Dynamics of PB2 degradation

Production of PB2-degrading enzyme present in *A. fumigatus* culture filtrate is presented in Fig. 2B. The obtained results showed that enzymatic activity was maximal after 36 h of culture in liquid medium and decreased thereafter over time. The culture filtrate from this time-point was therefore used as enzyme source for PB2 degradation. The products formed after enzymatic treatment of PB2 were analyzed. Fig. 3A shows the HPLC elution profile of PB2 (17.2 min) and its corresponding mass spectrum (*m/z* 577). After enzymatic reaction with extracellular culture from *A. fumigatus*, a major peak named PB2-X with a retention time (RT) of 10.5 min was observed with the corresponding mass spectrum of the purified peak, *m/z* 609 (Fig. 3B).

3.3. Characterization of PB2-X

In order to understand the different steps involved in the degradation of aromatic compounds, it is essential to identify the products formed. Knowledge of substrates and products results in the ability to understand reaction mechanisms and search for key enzymes involved. Assays for their identification are commonly followed by chromatographic and mass spectrometric analysis. The identification of the major product formed, was based on the analysis of the PB2-X fraction (RT of 10.5 min) following HPLC separation. The change in RT observed was consistent with an increase in polarity. The MS spectrum of this compound showed an ion at *m/z* 609 that corresponded to the pseudo-molecular ion of PB2-X (Fig. 3B). For comparison, pseudo molecular ion of PB2 is at *m/z* 577 in negative mode (Fig. 3A). The mass increase (32 Da) suggested that PB2 was initially attacked by an oxygenase (present in the 36 h culture filtrate). Fan et al. [40] in

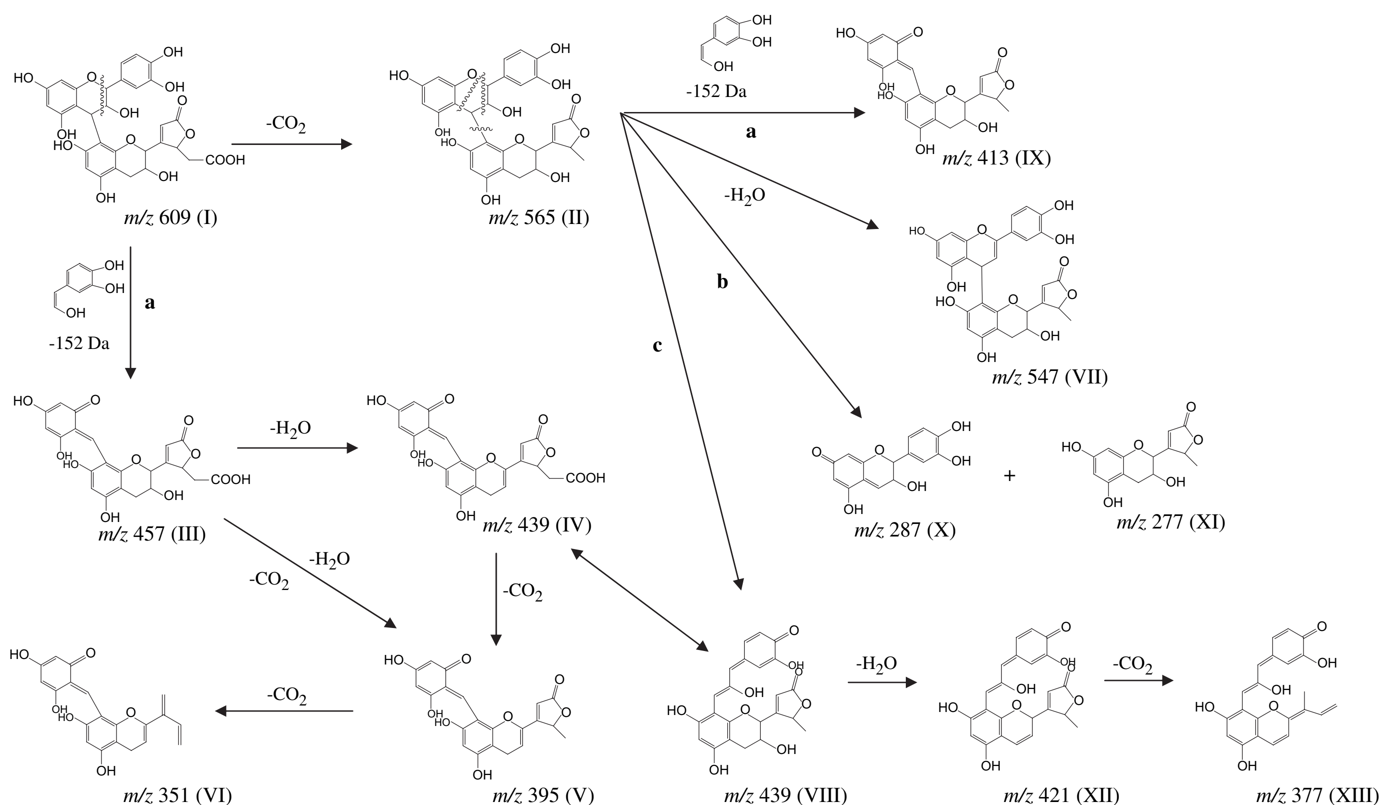
their recent study of oxidative derivatives of monomeric catechin in grape seeds, observed a similar mass increase. It is commonly accepted that *o*-cleavage or *m*-cleavage of aromatic rings after oxygen incorporation into the molecule follows an oxidative process in the presence of oxygenase enzymes [41]. This constitutes a major pathway by which aromatic compounds are degraded in the environment. To determine the structure of PB2-X and to localize the catechin moiety involved in oxidative degradation, the peak with RT of 10.5 min was purified by HPLC and analyzed by multiple fragmentation mass spectrometry (MS^n).

3.4. MS^n fragmentation pattern of PB2-X (ion at m/z 609)

The structure of PB2-X was characterized by studying the fragmentation scheme of its pseudo molecular ion at *m*/*z* 609. MS fragmentation of PCs is well documented. They can result in (a) the loss of ring B through retro-Diels–Alder (rDA) fission [42]; (b) quinone methide formation from direct cleavage of interflavan bond [32]; and (c) loss of ring A by heterocyclic ring fission [43]. Scheme 1 shows the different fragments obtained from MS^n of the pseudo molecular ion at *m*/*z* 609 (PB2-X). Five fragment ions (II–VI) were observed at *m*/*z* 565, 457, 439, 395 and 351. On MS^2 analysis, the loss of 44 by decarboxylation produced the fragment at *m*/*z* 565. Besides, the cleavage of the C ring of the extension unit (upper unit) through rDA led to the ion at *m*/*z* 457. The rDA cleavage was preferentially observed on the extension unit and was consistent with previously published results indicating that this position is energetically favored [32]. The fragments at *m*/*z* 439, 395 and 351 were generated from the fragmentation of the ion at *m*/*z* 457, itself coming from ion at *m*/*z* 609 (MS^3 experiment). Water elimination, loss of H_2O/CO_2 and subsequent elimination of CO_2 corresponded to ions at *m*/*z* 439, 395 and *m*/*z* 351, respectively. MS^n structure analyses were consistent with the action of an oxygenase on the catechol ring of the terminal unit of PB2. These first set of studies indicated that the extension unit (Fig. 1B) was not modified. The possible fragmentation pathway of these ions is shown on the left side of Scheme 1.

The MS^2 ion at *m*/*z* 565 (Scheme 1) was further fragmented (MS^3) producing seven other fragment ions (VII–XIII) at *m*/*z* 547, 439, 413, 287, 277, 421 and 377. Scheme 1 presents the possible fragmentation pathway resulting from MS^3 study of the ion at *m*/*z* 565, itself coming from the MS^2 fragmentation of *m*/*z* 609. Water elimination produced the ion at *m*/*z* 547 whereas the ion at *m*/*z* 413 was formed by rDA fission. A direct cleavage of interflavan bond between extension unit and terminal unit of the ion at *m*/*z* 565 produced the fragments at *m*/*z* 287 and 277. However, another ion at *m*/*z* 439 was also observed. This indicated a loss of 126 Da corresponding to ring A elimination after heterocyclic ring fission. Finally, the ions at *m*/*z* 421 and 377 were formed from H_2O and CO_2 elimination, respectively.

As indicated earlier, the main fragments from PB2-X observed in MS^2 and MS^3 experiments were wholly consistent with an enzymatic oxidative degradation of the B ring of the



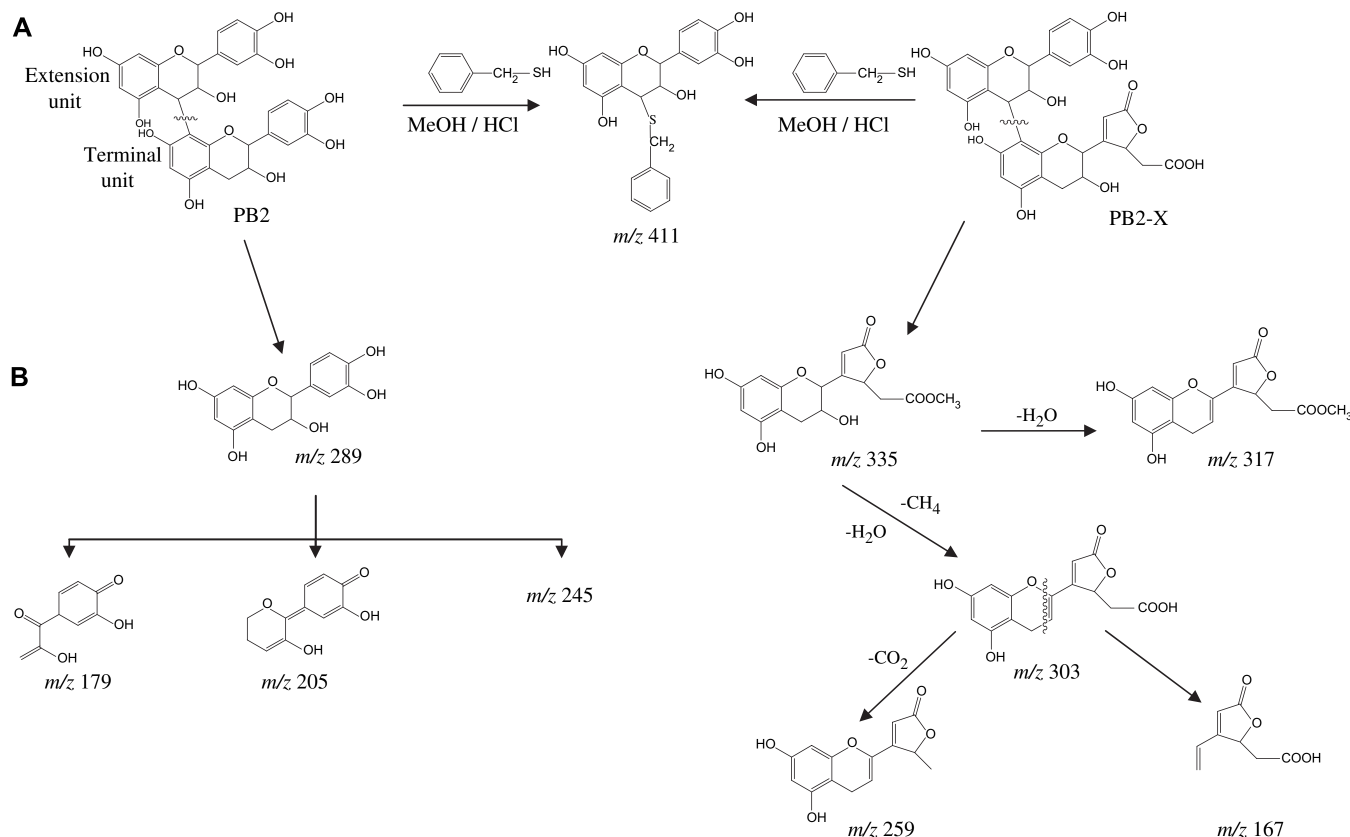
Scheme 1. Proposed fragmentation pathway from MS^3 study of the ion at *m*/*z* 609: (a) loss of ring B through rDA fission; (b) direct cleavage of interflavan bond; and (c) loss of ring A by heterocyclic ring fission.

terminal unit. Nevertheless, in order to corroborate these results, PB2 and PB2-X were subjected to thiolysis and the products were analyzed by HPLC–UV–MS.

3.5. MS fragmentation study of the thiolysis reaction products of PB2 and PB2-X

Freeze-dried samples of PB2 and PB2-X were subjected to the thiolysis reaction as described by Guyot et al. [23]. The reaction products were then analyzed by HPLC coupled with UV and MS detection. The thiolysis reaction has been used frequently for the characterization of procyanidins. It corresponds to the acidic cleavage of the interflavan linkage of procyanidins. Terminal units are liberated in their free epicatechin form whereas extension units are liberated as the corresponding benzylthioether adduct [22]. As expected, analysis of the thiolysis products of PB2 (Scheme 2) revealed two reaction products (peak 1, RT = 19.3, *m/z* 289 and peak 2, RT = 42.2, *m/z* 411). These two products were thus identified as (−)-epicatechin and (−)-epicatechin benzylthioether. A fragmentation of the ion at *m/z* 289 produced the pseudo molecular ions described in other studies [44,45], which were in accordance with the structure of PB2. When PB2-X was subjected to the thiolytic reaction two chromatographic peaks were observed. One peak (RT = 42.2 min, *m/z* 411) was easily identified as (−)-epicatechin-benzylthioether indicating that the extension unit of PB2-X corresponded to an epicatechin unit. It was therefore confirmed, following MS^n studies that the extension unit (upper moiety) of PB2-X was not modified by the enzyme extract from the *A. fumigatus* culture filtrate. The other peak (RT = 21.1 min) that corresponded to the terminal unit after thiolytic reaction, gave a pseudo molecular ion at *m/z* 335. By comparison with previous works dealing with the oxidation of monomeric catechin [40] and as shown in Scheme 2, the reaction product was identified as the methylated form of the oxidative derivative of epicatechin. Methylation of the carboxylic group can occur during thiolysis because reaction conditions (HCl + MeOH) are favorable to esterification by methanol. Similar (unmethylated) oxidation derivatives of epicatechin have been structurally elucidated [40]. As shown in Scheme 2, the MS^2 fragmentation pattern of the *m/z* 335 pseudo molecular ion (Fig. 4) was consistent with the proposed structure for the thiolysis product. Fan et al. identified similar molecules termed viniferone B or C [40] or (−)-epicatechin lactone [46]. In order to confirm lactone formation during the biodegradation of PB2 to PB2-X, both substrate and product of enzymatic treatment were subjected to infrared spectroscopy.

Fig. 5 shows the FT-IR spectra of PB2 and PB2-X. The spectral profile of both compounds are extremely similar, except for the presence of a strong band from PB2-X sample at 1724 cm^{-1}, attributed to the C=O stretching which may be assigned to a lactone group [47]. This band was not observed in IR spectra of PB2. From these data, it is reasonable



Scheme 2. (A) Thiolysis reaction from PB2 and PB2-X. (B) Proposed MS fragmentation of the terminal units (pseudo molecular ion at *m/z* 289 and 335) formed from thiolysis reaction of PB2 and PB2-X.

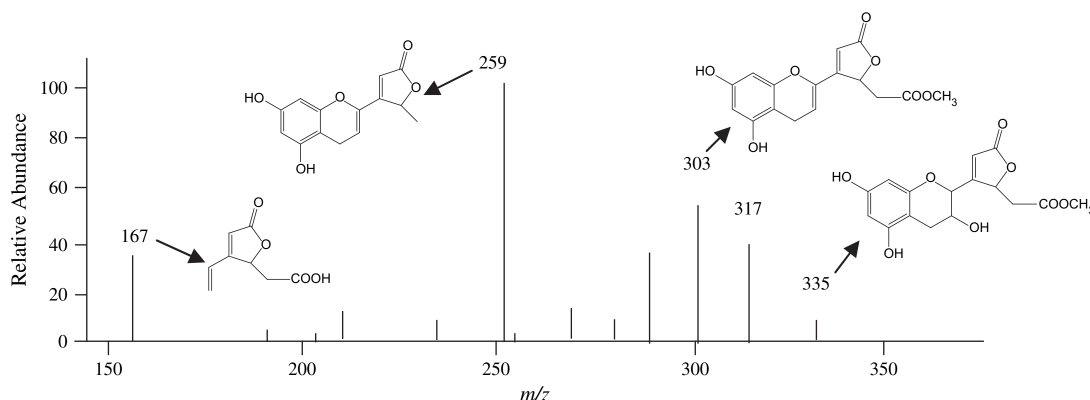


Fig. 4. MS^2 spectrum of the pseudo molecular ion at *m/z* 335 corresponding to a thiolytic reaction product of PB2-X. The ions generated are at *m/z* 317, 303, 259 and 167.

to propose that the lactone was formed following procyanidin B2 oxygenation. These results confirm the oxygenation of PB2 dimer with a subsequent lactone formation. It is uncertain at this point whether lactone formation was spontaneous or whether an enzyme present in the culture filtrate was responsible for its formation. However, Mendel et al. [48] did observe that an extradiol dioxygenase enzyme was able to catalyse an intra-molecular lactonisation reaction upon its natural substrate. Enzyme purification studies are presently underway to answer the question.

3.6. Consumption of oxygen

Oxygen consumption could therefore be associated to the biodegradation of PB2 dimer due to oxidation of this substrate in presence of an oxygenase enzyme. An experiment of oxygen consumption was performed using a Clarke oxygen electrode. After 5 min of oxygen saturation of PB2 (1 mM), a sample of extracellular enzyme from 36 h of culture was added to the electrode. An oxygen consumption of about 80% was observed within 30 min (Fig. 6) corresponding to 0.8 mmol of O_2 consumed·per liter per hour. This confirmed the presence of an oxygenase. In addition, no oxygen consumption was observed with boiled extracellular enzyme extracts.

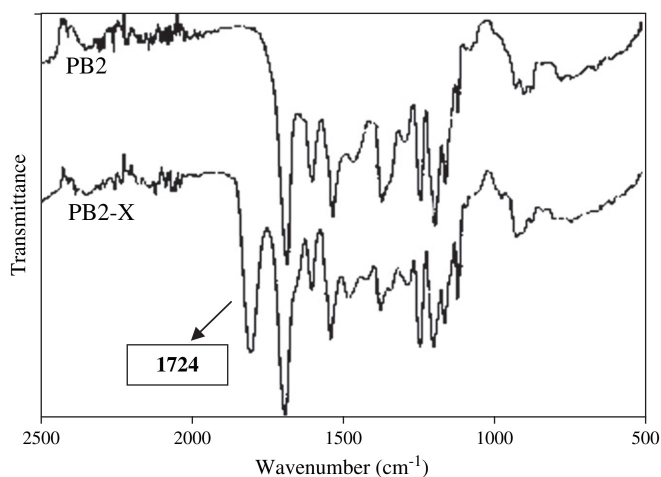


Fig. 5. FT-IR spectra of procyanidin B2 (PB2) and biodegraded procyanidin B2 (PB2-X).

3.7. Possible degradation pathway from PB2 to PB2-X

The extracellular enzymatic extract of *A. fumigatus* contains a number of proteins with potential activities on PB2. The data indicate that the initial attack is on the catechol ring of the terminal unit, through an oxidation process. The di-ol groups could be oxidized to dicarboxylics as shown in Scheme 3. Similar di-carboxylics structures were postulated by Fan et al. [40], who also propose a hydration step prior to lactone formation. A recent review by Bhat et al. [11] stresses that microbial degradation of PCs usually takes place through the cleavage of the heterocyclic ring. This was not the case in the present study. However, Loh and Chua [49] demonstrated that in *Psuedomonas putida* culture, bacterial catechol 1,2-dioxygenase enzyme oxidized catechol to *cis*, *cis* muconate *via ortho* cleavage. In addition, a second enzyme, cyclo-isomerized this product through a lactonizing enzyme. In the present study, the enzyme purification process will enable us to decipher the different steps involved in the initial production of PB2-X.

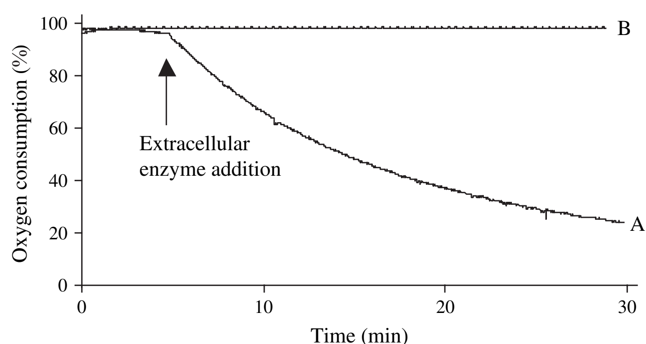
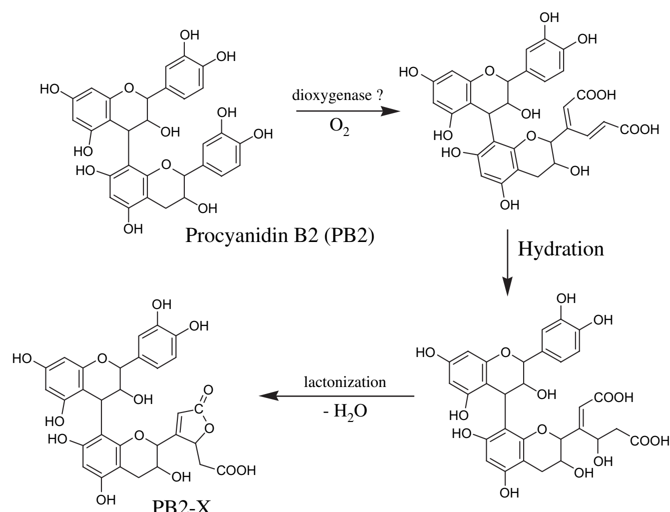


Fig. 6. Utilization of oxygen in the presence of extracellular enzyme produced by *A. fumigatus* using PB2 as substrate. Addition of extracellular enzyme extract (A), addition of boiled extracellular enzyme extract (B).



Scheme 3. Possible pathway of procyanidin B2 (PB2) degradation by *A. fumigatus*.

4. Conclusions

A. fumigatus is able to metabolize apple-purified PC, growing preferentially on PC oligomers. This study was followed by the identification, for the first time, of a product of fungal PC degradation. The structure was deduced from detailed analysis of multiple MS fragmentation patterns and thiolysis degradation products. The characteristics of the ion at *m*/*z* 609 indicated that in the presence of an extracellular extract from *A. fumigatus* strain, procyanidin B2 dimer underwent biodegradation into [(−)-epicatechin-(4β-8)-(−)-epicatechin lactone]. The reaction resulted in the opening of the catechol ring of the terminal unit of the dimer *via* intra-diol cleavage by an oxygenase enzyme. This study constitutes the first step towards the elucidation of the degradation pathway of procyanidins by filamentous fungi, paving the way in the use of novel tools (enzymes) to understand procyanidin degradation.

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