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Biotransformation of procyanidins by a purified fungal dioxygenase: Identification and characterization of the products using mass spectrometry

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ABSTRACT

Procyanidins commonly known as condensed tannins are a type of polyphenol with wide abundance naturally. They are commonly known as potent anti-oxidants with powerful free radical scavenging activity as well as anti-tumor-promoting activity. Little is known about the enzymatic mechanisms/pathways involved in the microbial biotransformation of these polyphenolic molecules. The extracellular enzyme, dioxygenase produced by *Aspergillus fumigatus* was used as *in vitro* tools to study the degradation pathway of a model procyanidin dimer, namely procyanidin B2. The enzyme was purified to homogeneity by a two step process of anion-exchange chromatography coupled with FPLC followed by gel-filtration chromatography coupled with HPLC and the molecular mass estimated. In addition, the different biotransformed products resulted from the dioxygenase action on PB2 were purified using Reversed-Phase-High Performance Liquid Chromatography prior to their identification and characterization by structural elucidation using Electrospray Ionization-Mass Spectrometry. Subsequently, the mechanism of dioxygenase action on procyanidin dimer was defined.

1. Introduction

Procyanidins (PCs) or condensed tannins are phenolic compounds with pronounced biological activities found in many plants, in wide variety of fresh fruits, foods and beverages [1]. PCs are known for their ability to bind strongly to proteins, reducing significantly the nutritional value of the feed especially in animal diets [2]. Recently, procyanidins have attracted interest because of their antioxidant properties [3,4]; also increasing evidence for oligomers in a wide range of active assays has attracted attention to their structural elucidation [5]. Owing to the complexity of the oligomers, most studies have focused on specific dimeric procyanidins. PCs are known to inhibit microbial growth [6]. Nevertheless, certain microorganisms mainly bacteria are able to grow on procyanidins by using it as a sole carbon source [7]. However, only the information on PCs degradation by fungi is scarce mainly due to lack of commonly available substrates. Mean time, there are certain reports mentioning the ability of filamentous fungi to degrade these complex molecules [8,9]. Moreover, the enzymatic mechanisms have not been established as standards are scarce and expensive. Also, little is known about the enzymes involved in the degradation pathway of these oligomeric procyanidins. The ability to modulate or biotransform these molecules could have significant applications in the field of food biotechnology (fruit juice clarification, control of palatability, digestibility of feedstock, etc.).

Natural PCs are oligomers and polymers of flavan-3-ol units such as (+)-catechin or (−)-epicatechin [10] mostly linked by C4-C8 or C4-C6 inter-flavan bond (Fig. 1). In 1994, Nguz et al. [11] reported for the first time the degradation of PCs by *Penicillium*. PCs are characterized by their average degree of polymerization (DP_n) that corresponds to the average number of flavanol units making up the polymer and it is measured as the molar ratio between all units (terminal + extension) and terminal units. The ratio obtained after procyanidin de-polymerization in the presence of benzyl thioether (thiolysis) can be quantified by HPLC analysis of the thiolysis medium [12,13]. Also, the use of thiolysis-based technique has allowed the characterization and quantification of the constitutive units of procyanidins in apples and apple products [14]. In recent years, the coupling of liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization (ESI) techniques has been proven to be a powerful tool for the characterization of PC structures. Once identified, PC structures can be elucidated through the study of fragment ions obtained by multiple fragment mass spectrometry (MS^n) [15]. In addition, more recently, LC–MS

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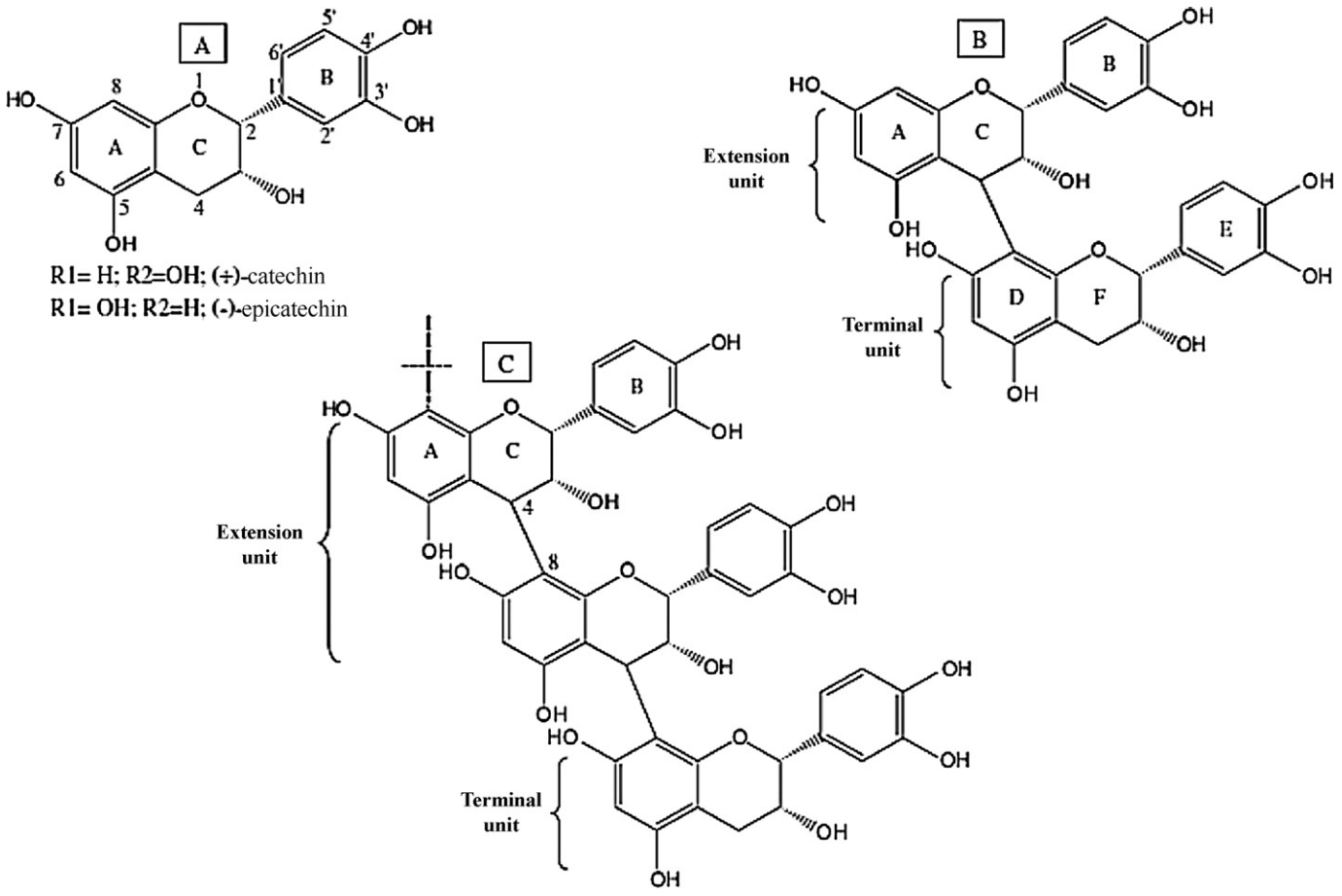


Fig. 1. General structures of procyanidins: (A) (+)-catechin and (−)-epicatechin monomers, (B) procyanidin B2 dimer, (C) proyanidin oligomers with C4-C8 linkage (*adapted from* Contreras-Domínguez et al., 2006).

and LC–MS–MS was shown to be well adapted to the analysis of polyphenol oxidation products resulting from chemical or enzymatic oxidation [16]. Also, there are reports stating that catechol 1,2-dioxygenase and catechol 2,3-dioxygenase are some of the key enzymes involved in the catabolism of monocyclic aromatic compounds [17]. In a previous research work [18], the main degradation product resulting from the action of an oxygenase from *Aspergillus fumigatus* on procyanidin B2 was identified.

The aim of the present study was to obtain a complete understanding of the different steps involved in microbial degradation of condensed tannins (PCs) through the study of key enzymes involved in the degradation process. The extracellular enzyme, dioxygenase produced by *A. fumigatus* was used as *in vitro* tools to study the degradation pathway of a model procyanidin dimer, namely procyanidin B2 [(−)-epicatechin-(4β-8)-(−)-epicatechin]. The enzyme was purified to homogeneity and the different biotransformed products obtained after the catalytic action of the enzyme on Procyanidin B2 (PB2) dimer were purified using liquid chromatography, subsequently their structures were elucidated by electrospray ionization-mass spectrometry (ESI-MS) and finally the mechanism of enzyme action was defined.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The filamentous fungus *A. fumigatus* MC 8 (wild strain isolate, IRD Laboratory culture collection, France) was cultured on Potato Dextrose Agar (Sigma) at 30 °C for spore production. Six days old spores were harvested with 30 mL of sterile distilled water containing 0.01% (v/v) Tween 80 under controlled agitation. The spore suspension (5×10^7 spores/mL) obtained was used as inoculum of culture medium.

2.2. Culture medium

Solid-State Culturing (SSC) was employed for production of the enzyme. Zero point three (0.3) gram of Polyurethane foam (PUF) was weighed into 100 mL Erlenmeyer flask, cotton plugged and sterilized. Nutrient (basic) solution [19] was prepared by dissolving (in g L^{-1}) $(NH_4)_2SO_4$ 1.0; KH_2PO_4 1.3; Na_2HPO_4 0.12; $MgSO_4$ 0.3; urea 0.3; glucose 2.0 in distilled water, supplemented with 0.1% of oligo-element solution ($FeSO_4{\cdot}7H_2O$ 5.0; $MnSO_4{\cdot}H_20$ 1.6; $ZnSO_4{\cdot}7H_2O$ 1.4; $CaCl_2$ 2.0); prepared in distilled water. The pH of the oligo-element solution was adjusted to 6.0 using NaOH (3.0 M) and sterilized at 121 °C, 15 psi for 20 min.

2.3. Induced enzyme production

PCs with DP*n* of 9 were obtained from a cider apple freeze-dried powder (*Marie Ménard* variety) by aqueous acetone and solid phase extraction [20]. This was used as a carbon source for *A. fumigatus* growth and to induce the dioxygenase enzyme production. Purified PCs dissolved in sterile nutrient (basic) solution were added aseptically to the PUF (solid support) using a syringe filter (PVDF- 0.22 μm, Millipore) to reach a final concentration of 2.0 g L^{-1}. Inoculation was done with *A. fumigatus* spore suspension (respecting the final moisture content −85 ± 2%). The flasks were then shaken (in upright position, tapped at the bottom) gently to homogenize the culture medium. Fermentation was carried out at 30 °C for 36 h. All of the experiments were performed under strict sterile conditions and in triplicate.

2.4. Extracellular extract

The fermented medium was mixed gently using a sterile spatula and the contents were squeezed through a sterile syringe (50 mL, Millipore) using hand pressure to get the extracellular enzyme extract. The extract was collected in clean storage vials, a portion of it stored at 4 °C was used for enzymatic as well as other assays and the remainder was frozen (at −80 °C) until further use.

2.5. Procyanidin removal from extracellular extract

The presence of non-biotransformed (residual) PCs in the extracellular enzyme extract is responsible for interferences with different types of assays. For instance, it

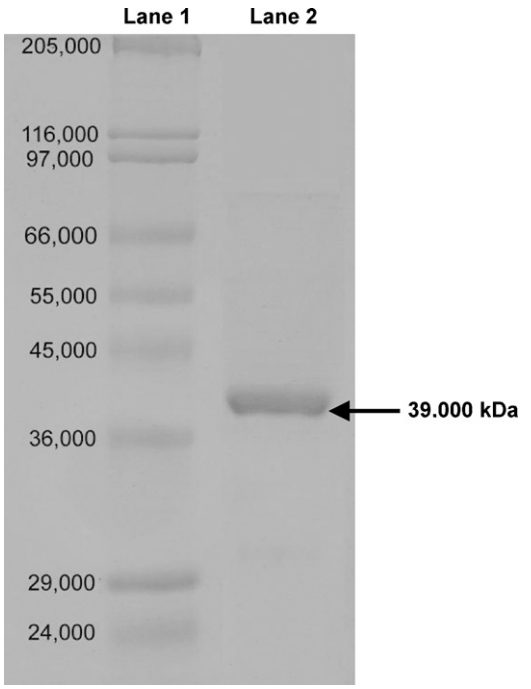


Fig. 2. SDS-PAGE of purified dioxygenase from *A. fumigatus* MC8. Lane 1: standard proteins of different molecular weight (205.0–24.0 kDa); Lane 2: purified (freeze-dried sample after gel filtration) dioxygenase run in SDS-PAGE (10%) and stained with commassie blue.

leads to heavy non-specific interactions with chromatographic materials (especially solid phases) during enzyme(s) purification processes. As an attempt to exempt the residual procyanidins from the extracellular extract, ampholyte (Bio-Lyte® 3/10, Bio-Rad) was added to the crude enzyme extract (0.24%, v/v) with the objective of precipitating selectively procyanidins and at the same time retaining enzyme activity. The mixture was kept at 4 °C for 1 h and then centrifuged at 12,000 rpm for 20 min. The supernatant was collected and assayed for dioxygenase activity. The total procyanidin content of the extracellular enzyme extract before and after the addition of ampholyte solution was also quantified.

2.6. Enzyme assay

The dioxygenase activity was estimated by the ability of the extracellular enzyme extract to degrade/bio-transform the specific dimeric substrate procyanidin B2 (PB2) [Fumakoshi, Japan]. The assay mixture contained 50 μL of PB2 solution (1.0 mM), 100 μL of phosphate buffer, pH 6.0 (0.2 M) and 100 μL of the extracellular enzyme extract. The reaction mixture was vortexed and incubated at 30 °C for 60 min. Ten % of TriChloro Acetic acid (TCA) in water was used to stop the reaction. The assay mixture was then centrifuged (micro-centrifuge, Millipore) at 15,000 rpm for 10 min. The supernatant was passed through micro filter (0.22 μm, PTFE, Millipore) into small glass vials for High Performance Liquid Chromatography (HPLC) analysis. The dioxygenase activity was determined by HPLC by following the disappearance of the PC dimer PB2 (peak) at 280 nm. A substrate blank was also prepared and analyzed against the reaction mixtures. Standard concentrations of PB2 from 1.0 to 5.0 mM were also prepared and analyzed in the HPLC. One unit of activity was defined as the amount of enzyme that catalysed the disappearance of 1 μmol min^{-1} of PB2 at 30 °C.

2.7. Total procyanidin determination

Total PCs were estimated as reported by Gessiner and Steiner [21]. The reaction mixture contained 7.0 mL of butanol–HCl taken in a glass tube (15 ml), with 500 μL of extracellular extract added to it. The reaction mixture was then heated at 95 °C for an hour and 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.

2.8. Bradford (micro) assay for total soluble protein content

The total soluble protein content of the extracellular extract before and after the addition of ampholyte was quantified by using the method of Bradford [22]. The extracellular extract (100 μL) was diluted to a final volume of 1000 μL using Milli-Q ultrapure water to which 200 μL of concentrated Bradford dye (Bio-Rad) was added. The solution mix was vortexed well and incubated at room temperature for 30 min. Thereafter, the samples were measured at 595 nm against a reagent blank (containing procyanidins) in a spectrophotometer. Several dilutions of protein standard (Bovine Serum Albumin, *Sigma*) from 1 to 25 μg mL^{-1} concentration were also prepared and read at the same wavelength. The OD_{595} versus concentrations of standards plotted, served as the standard curve in determining the unknown concentrations.

2.9. Iso-electric focusing (IEF)

The pI of the enzyme was determined by using the technique of iso-electric focusing. Forty milliliters of the extracellular enzyme extract mixed with 1.0 mL of ampholyte (3/10, Bio-Rad) solution was loaded into Rotofor [Preparative Iso-Electric Focusing cell (IEF); Bio-Rad] system under controlled manner. A power pack connected to the system delivered 12 W to the IEF cell. The whole unit was connected to a cooling system and was maintained at 4 °C during the entire run. The concentrated and separated samples were harvested as (2.0 mL × 20) different fractions from the IEF cell using a harvesting kit under vacuum. The pH of each of the fractions obtained was read immediately. Subsequently, the fractions were assayed for dioxygenase activity as well as the total soluble protein content.

2.10. Purification of extracellular dioxygenase

All steps in the purification were carried out at 0–4 °C unless otherwise mentioned. Chromatography columns were carried out using Fast Protein Liquid Chromatography (FPLC) system [Bio-Rad, Germany] as well as High Performance Liquid Chromatography system [Agilent Technologies, USA]. The supernatant obtained after procyanidin precipitation from the extracellular extract using ampholyte (3/10) solution was applied to UNO™ Q6 anion-exchange column (Bioscale™ Mini UNOsphere™ Q cartridge – 5.0 mL, BioRad) pre-equilibrated with 0.1 M acetate buffer, pH 5.0 (buffer A) and extensively washed using the same buffer. Proteins in the column were eluted using a linear NaCl gradient up to 2.0 M in buffer A using BioLogic DuoFlow FPLC system (BioRad). Fractions with dioxygenase activity were pooled, desalted by dialyzing against Milli-Q water at 4 °C and freeze-dried (Heto Power Dry LL1500 Freeze Dryer, Thermo Electron Corporation, U.S.A.).

2.10.1. Determination of molecular mass

The molecular mass of the purified protein was estimated by gel filtration chromatography using Bio-Sil® SEC-125 gel filtration column coupled with HPLC. The freeze-dried samples (obtained after anion-exchange chromatography) were re-suspended in Tris–NaCl buffer, pH 8.0 (0.1 M NaCl in 25 mM Tris) and loaded on to Bio-Sil® SEC-125 gel filtration column (300 mm × 7.8 mm) using Agilent HPLC system (Agilent Technologies, U.S.A.) pre-equilibrated with Tris–NaCl buffer, pH 8.0. Proteins were eluted with an isocratic elution using Tris–NaCl buffer, pH 8.0. Fraction(s) were purified from HPLC with respect to appearance of the absorption peaks at 280 nm for proteins. The fraction (peak) with dioxygenase activity was separated, desalted (as described above) and freeze-dried. The subunit molecular weight of the protein was determined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis using 10% (v/v) polyacrylamide gels was performed as described by Laemmli [23].

2.11. Reversed-phase HPLC analysis

The dioxygenase activity was quantified using HPLC by following the disappearance of the PC dimer PB2 at 280 nm. The HPLC apparatus was equipped with a HP quaternary gradient pump 1200 series (Agilent Technologies, U.S.A.), an autosampler and a photo diode array detector. The enzymatically cleaved products were separated on an Atlantis™ dC_{18} column (250 mm × 4.6 mm, Waters Co., U.S.A.). A binary gradient was applied (eluent A (%): acetic acid (2), Water (98); eluent B (%):

Table 1
Purification scheme for dioxygenase from *Aspergillus fumigatus* MC8.

Fraction	Volume (mL)	Protein (mg/mL)	Specific activity (Units/mg)	Purification fold	Recovery/yield (%)
Extracellular (crude) extract	75.0	2.77	1.37	1	100
Extracellular extract (*after PC precipitation using ampholyte*)	74.3	2.91	1.51	1	92.0
Eluate UNO™ Q column	5.0	3.35	11.53	8.4	83.3
Eluate Bio-Sil® SEC-125 column	2.5	0.73	53.0	38.7	35.2

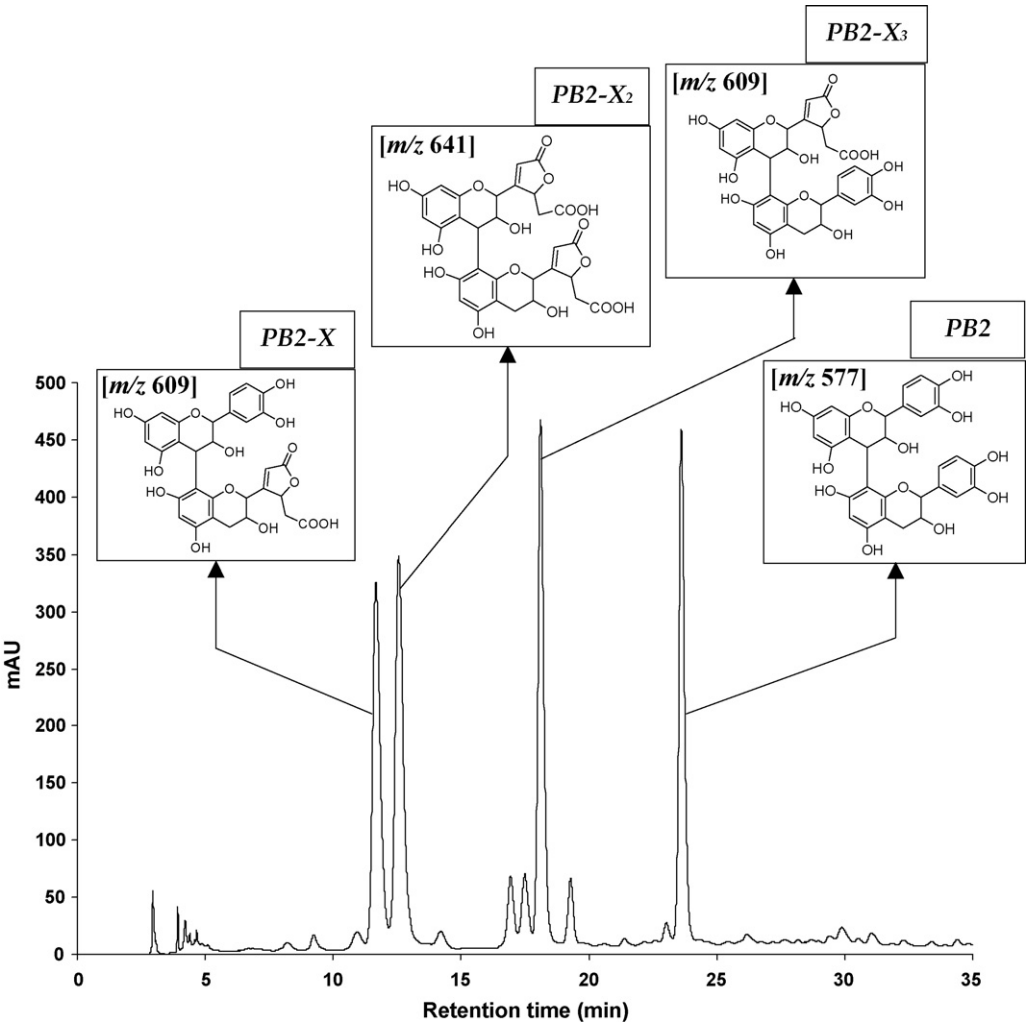


Fig. 3. UV 280 nm trace of the Reversed-Phase HPLC showing PB2 and its biotransformed products together with their elucidated structures (with their corresponding pseudo-molecular ion *m*/*z* values in the negative mode mass spectrometry).

Acetonitrile (100); initial, 7% B; 0–15 min, 45% B linear; 15–20 min, 50% B linear; 20–40 min, 100% B linear, at a flow rate of 1.0 ml min^{-1}, followed by reconditioning and recalibration of the column. Data were processed by using Chemstation® 2D software.

2.12. Purification of PB2 and the biotransformed products for analytical purposes

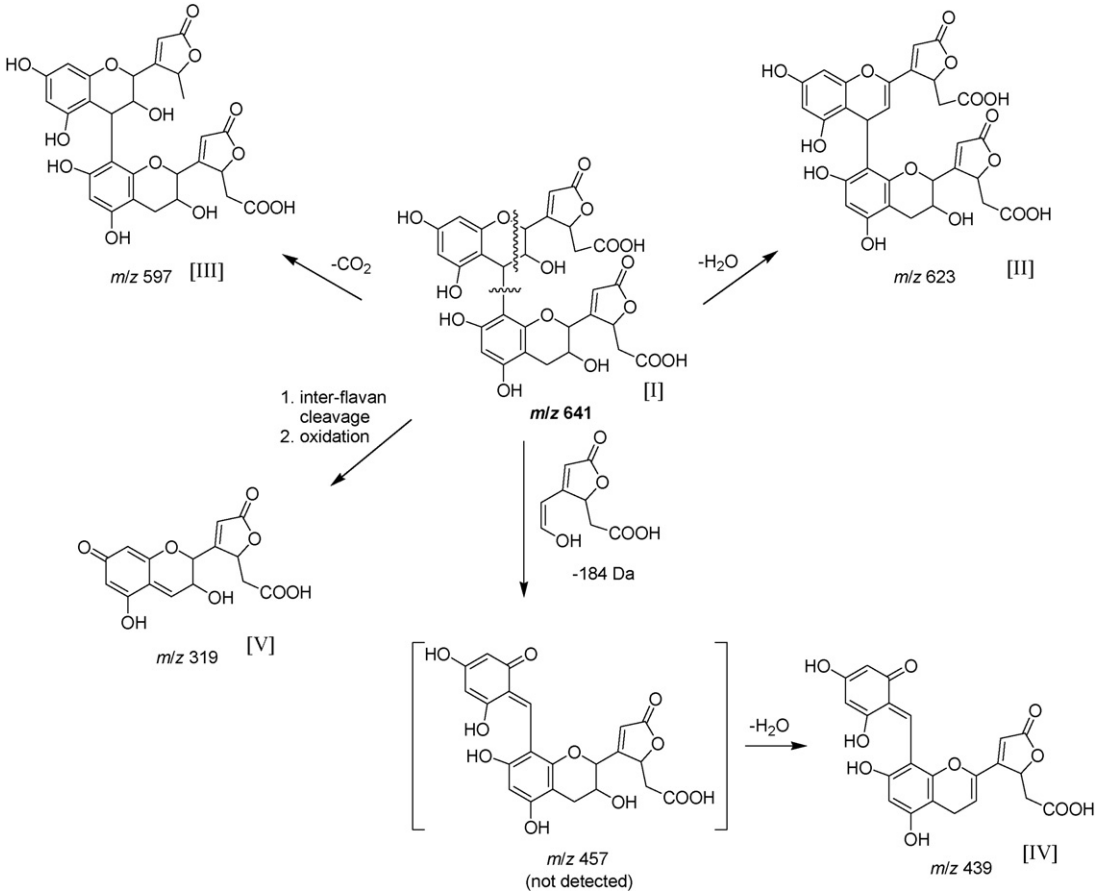
Purification of PB2 and the enzymatically cleaved products were performed by HPLC. The running and elution conditions were identical to those described above. Peaks corresponding to residual PB2 and those of the major degraded products obtained were collected separately (in total 4 peaks). Samples from about 50 runs in HPLC were collected to obtain sufficient quantity of the products used for analytical purposes. For each of the runs 3.0 μg of biotransformed PB2 were injected into HPLC column. Excess acetonitrile and acetic acid were eliminated by rotaevaporation at 35 °C and the residues were freeze-dried. The samples were then dissolved in MeOH (methanol) and filtered through a 0.45 mm Teflon membrane (PTFE, Millipore) and analyzed by Reversed-Phase High-Performance Liquid Chromatography/Electrospray Mass Spectrometry (RP-HPLC-ESI-MS). In addition, these samples were also subjected to thiolysis and the products were analyzed by RP-HPLC-ESI-MS.

2.13. HPLC-ESI-MS analysis of the biotransformed and thiolyzed products of PB2

The mass spectrometry (MS) apparatus was an LCQ Deca ion trap spectrometer (Thermofinnigan, San Jose, CA, USA) 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 binary gradient Pump 1100 series (Agilent Technologies), autosampler (Surveyor, Thermofinnigan), Diode array detector (UV6000Lp, Thermofinnigan) and data were processed by Xcalibur® version 1.2 software. Samples were separated on a Zorbax Eclipse XDB C_{18} column (2.1 mm × 150 mm, 3.5 mm, Agilent Technologies, U.S.A.). 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 50–2000. Thiolysis was carried out according to the procedure described by Guyot et al. [24]. In brief, 2.0 mL of anhydrous MeOH were added to freeze-dried biotransformed PB2 samples (≈3.0 mg). The reaction was carried out in glass tubes (250 μL) as follows: 50 μL of sample mixed with 100 μL of benzylthioether solution (5% in anhydrous MeOH) and 50 μL HCl (0.4N) 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 (biotransformed), samples were diluted in anhydrous MeOH and thiolysis was carried out as described just above. The reaction was stopped by transferring samples to an ice bath. HPLC-ESI-MS analyses of the thiolyzed products were also carried out as described above.

3. Results and discussion

The microbial synthesis of procyanidin biotransforming enzyme was induced by growing *A. fumigatus* MC 8 on solid medium containing cider apple procyanidins (DP*n*: 9) for 36 h. The extra-cellular enzyme was extracted from the fermented medium, purified and this purified dioxygenase enzyme was used to study the biotransformation of procyanidin B2. The biotransformed products were analyzed by LC–MS with or without combination of thiolysis reaction.



Scheme 1. Proposed fragmentation pathway of the ion (*m*/*z*) 641 from MS^2 analysis.

3.1. Procyanidin removal from extracellular extract

Ampholyte solution (3/10) was found to be much efficient in removing the PCs (residual) present in the extracellular extract than the classical technique of polyphenol precipitation using polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP). PC precipitation was achieved by the addition of ampholyte solution, resulting in about 94% of its removal from the extracellular extract retaining 92% of the enzyme activity indicating that the co-precipitation of protein with procyanidins were much less. In the case of PVP or PVPP it is the stronger affinity of these insoluble molecules towards the polyphenols makes them precipitate from a mixture while, the underlying mechanism of polyphenol precipitation by ampholytes is not known and is yet to be discovered as this is the first report on it.

3.2. Dioxygenase purification

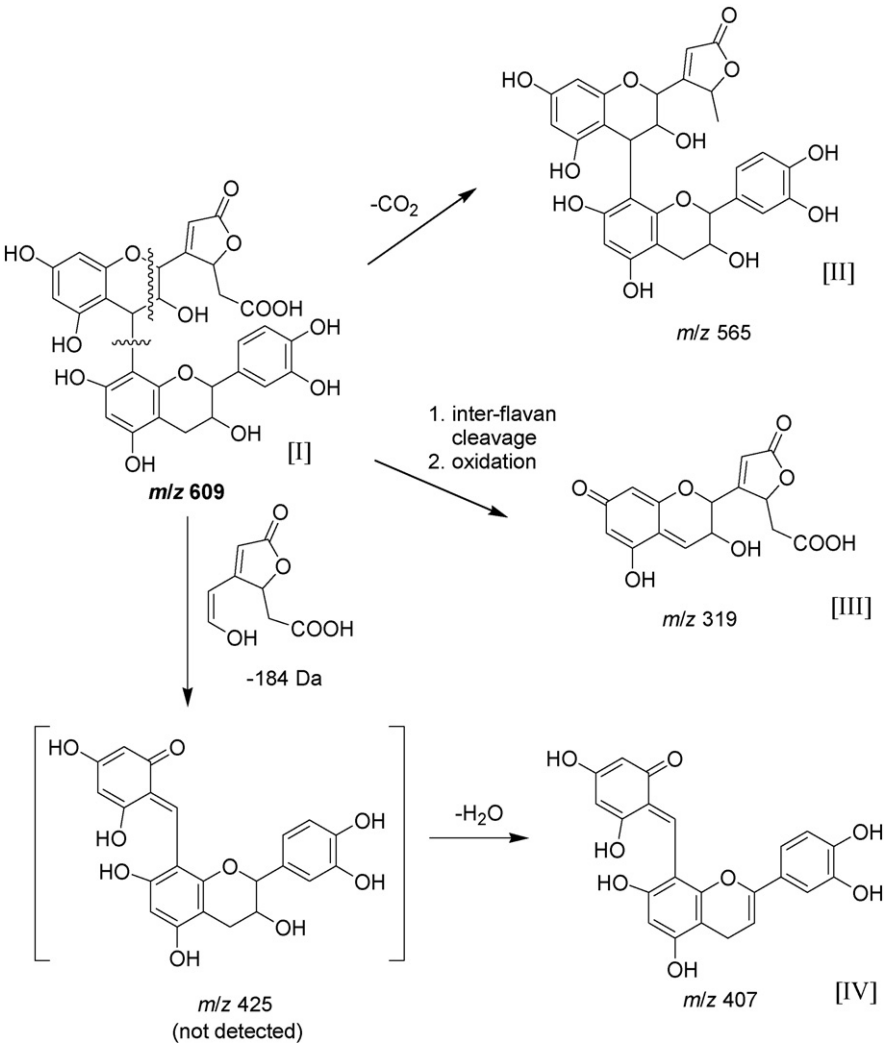
Iso-electric focusing using the system of rotofor resulted in obtaining the pI of the enzyme which was found to be 3.5. Hence, for further purification studies anion-exchange chromatography was selected. Out of six different fractions obtained from anion-exchange chromatography using Uno Q6 (5.0 mL) cartridge, only three fractions (over lapped peaks) exhibited dioxygenase activity. These three fractions were pooled together, desalted by dialysis against Milli-Q water (at 4 °C) and freeze-dried. The sample was then re-suspended in Tris–NaCl buffer (at 4.0 °C), pH 8.0 and run on HPLC-gel-filtration column to exclude out the proteins based on their size. The elution profile obtained was with a single peak clearly separated and eluted out at 6.2 min, followed by a bunch of peaks that eluted out of column after 8 min. The first eluted peak was found to have dioxygenase activity, separating the protein of our interest from rest of the contaminants (proteins). Hence, the first eluted peak (peak at 6.2 min) was purified, dialyzed and freeze-dried. The freeze-dried sample was dissolved in Tris–NaCl buffer and re-eluted on the same gel-filtration column to achieve homogeneity. Single peak was observed after gel filtration-HPLC indicating its homogeneity. A set of standard molecular weight protein markers (Bio-Rad's gel filtration standards, range: 1350 to 670,000 Da) were also injected into the gel filtration column to obtain the retention time (RT) of the standard proteins. In comparison with the standard protein markers, the molecular mass of the purified dioxygenase was estimated to be 117 kilo Dalton (kDa) under non-denaturing conditions. About 40-fold purification of the protein was achieved with a yield of 35% (Table 1).

3.2.1. Molecular mass

SDS-PAGE of the purified enzyme indicated the presence of a single band. The molecular weight of the purified dioxygenase was calculated to be ~39 kDa from the log plot obtained from the relative mobility of the standard proteins in SDS-PAGE (Fig. 2). However, molecular mass of the intact protein was found to be 117 kDa after gel filtration chromatography. This suggests that the enzyme is a trimer. This is the first report of a trimeric dioxygenase being purified from *A. fumigatus* MC8.

3.3. Characterization of the biotransformed products of PB2

The products formed after the enzymatic cleavage of PB2 by the action of dioxygenase were analyzed using HPLC. Chromatographic profile could be considered as a way to characterize the cleaved products: it informs on the polarity of the products (for



Scheme 2. Proposed fragmentation pathway of the ion (*m*/*z*) 609 from MS^2 analysis.

instance, in the present case with the enzymatically cleaved PB2, we can observe that all the cleaved products were eluted before PB2 indicating that they are more polar which is in accordance with their structures containing carboxyl groups). Fig. 3 shows the (UV 280 nm) HPLC profile of PB2 and the biotransformed products. After enzymatic reaction for 60 min at 30 °C, pH 6.0, with PB2 as substrate and the extracellular extract (enzyme) from *A. fumigatus*, four different (major) peaks were observed in the HPLC profile. Among them, three major peaks named PB2-X, PB2-X_2, and PB2-X_3 were eluted before PB2 showing RT at 11.6, 12.5 and 18.1 min, respectively (Fig. 3). They were purified by HPLC and analyzed by electrospray ionization-mass spectrometry. The corresponding molecular ions $[M-H]^-$ in the negative mode for each of these peaks were at *m*/*z* 609, 641 and 609, respectively. PB2 had a RT of 23.61 min with a corresponding molecular ion at *m*/*z* 577 (Fig. 3).

The major products obtained after the enzymatic cleavage of PB2: PB2-X, PB2-X_2, PB2-X_3 were identified and characterized by mass spectrometry (Fig. 3). The MS spectrum of the compound PB2-X showed an ion at *m*/*z* 609. This compound was already identified in a previous published work [18]. Compared to PB2 structure, PB2-X corresponds to a product with a structural modification of PB2 where the catechol ring of the terminal unit has been oxidized by the intra-diol dioxygenase action and finally converted into a non phenolic moiety corresponding to a (5-Oxo-tetrahydro-furan-2-yl)-acetic acid group (Fig. 3). For comparison, the MS spectrum of the pseudo-molecular ion of PB2 is at *m*/*z* 577 in negative mode.

The MS spectrum of compound PB2-X_2 showed a pseudo-molecular ion $[M-H]^-$ at *m*/*z* 641 (Fig. 3) corresponding to an increase of 64 atomic mass unit (amu) compared to the *m*/*z* of PB2 pseudo-molecular ion. This mass increase indicated that PB2-X_2 has integrated 4 additional oxygen atoms in its structure in comparison to PB2, which is in accordance with a dioxygenase intra-diol cleavage of two catechol rings. This suggested that both the terminal and the extension units of the dimeric molecule were oxidized. It could be noted that oxidation of the catechol ring into a (5-Oxo-tetrahydro-furan-2-yl)-acetic acid group can lead to the formation of a new asymmetric carbon which was not asymmetric before oxidation of the catechol group which means that two or four diastereoisomers for each of the compounds 641 and 609 could be potentially formed.

The full MS spectrum of compound PB2-X_3 was very similar to that of PB2-X, showing a pseudo-molecular ion at *m*/*z* 609 (Fig. 3). Nevertheless, its RT was clearly longer than that of PB2-X which indicated it is probably less polar. Therefore, our first hypothesis was that this compound was the isomeric form of PB2-X with an intra-diol catechol ring cleavage located at the extension unit of the dimer PB2 instead of at the terminal unit.

To determine the structures of PB2-X_2 and PB2-X_3 and also to localize the respective oxidized moieties involved in degradation,

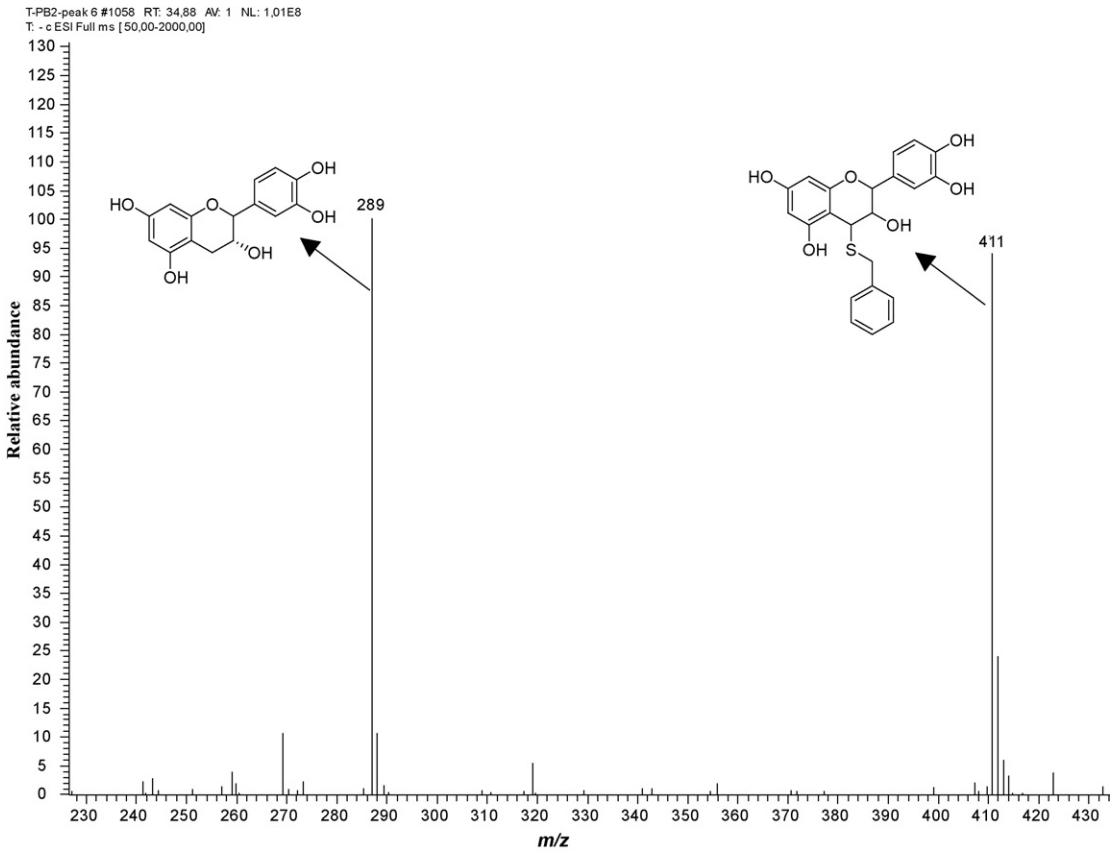


Fig. 4. MS spectrum of the thiolytic reaction product of PB2. The ions generated are at *m/z* 411 [(−)-epicatechin benzylthioether] and 289 [(−)-epicatechin].

these two peaks were purified by HPLC and analyzed by multiple fragmentation mass spectrometry (MS^n) and thiolysis.

3.4. MS^2 fragmentation study of the molecular ions of PB2-X_2 at *m/z* 641 and PB2-X_3 at *m/z* 609

Recently, Contreras-Domínguez et al. [18] showed the MS^n fragmentation scheme of PB2-X. Several fragmentation pathways were elucidated: (a) the loss of ring B through retro-DielseAlder (rDA) fission [25]; (b) quinone methide formation from direct cleavage of interflavan bond [26]; and (c) loss of ring A by heterocyclic ring fission [27].

Scheme 1 shows the different fragments obtained from MS^2 analysis of the pseudo-molecular ion of PB2-X_2 at *m/z* 641. Four main fragment ions (II–V) were observed at *m/z* 623, 597, 439 and 319. The loss of 44 by decarboxylation produced the fragment at *m/z* 597, while the loss of a water molecule led to the fragment at *m/z* 623. Besides, the cleavage of the C ring (oxidized) of the extension unit through retro-DielseAlder (rDA) fission led to the ion at *m/z* 457 (not detected), subsequently losing one water molecule from this fragment resulted in another fragment at *m/z* 439.

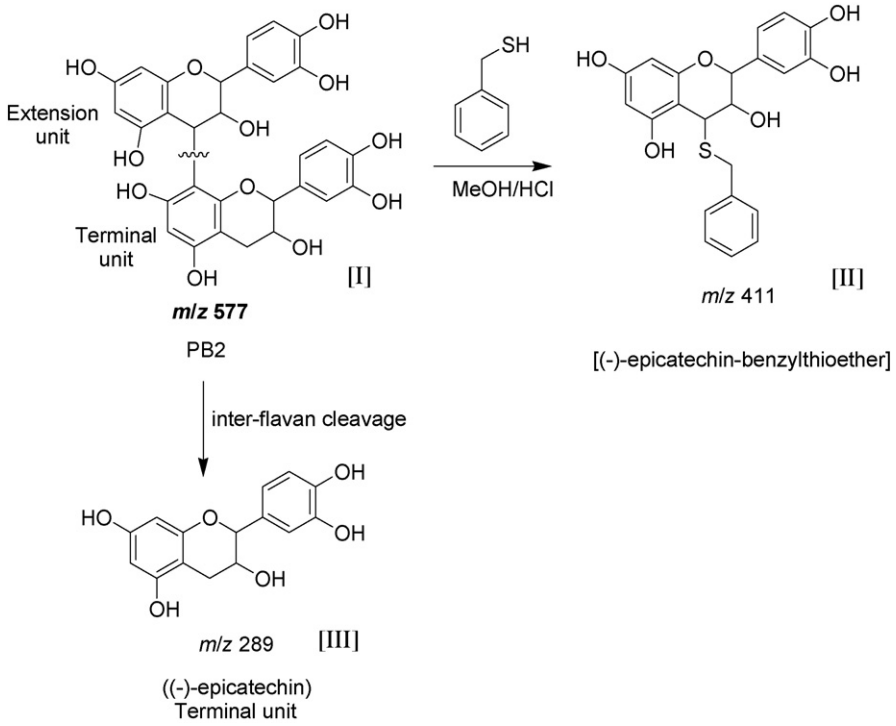
Scheme 2 presents the possible fragmentation pathway resulted from MS^2 study of the molecular ion of PB2-X3 at *m/z* 609. Three main fragment ions (II–IV) were observed at *m/z* 565, 319 and 407. The first one, with *m/z* 565 corresponded to a decarboxylation (loss of 44 amu) and was also observed for PB2-X [18]. Interestingly, the two other fragments (i.e. at *m/z* 319 and 407) were not observed on PB2-X MS^n spectra. A direct cleavage of the inter-flavan bond between extension unit and terminal unit of the ion at *m/z* 565 lead to the formation of the fragment ion at *m/z* 319 which is in accordance with the structure of extension unit that has been oxidized by the enzyme (Scheme 2).

Another ion at *m/z* 407 was also observed. This indicated a loss of 184 amu (atomic mass unit) corresponding to the B ring elimination of the extension unit as a consequence of retro-DielseAlder (rDA) fragmentation of the heterocyclic C-ring. The resulting fragment at *m/z* 425 (possible intermediate) was not detected probably because it is immediately converted into the fragment at *m/z* 407 after the loss of a water molecule.

The main fragments from PB2-X_2 & PB2-X_3 observed from MS^2 experiments were wholly consistent with an enzymatic oxidative degradation of the B ring (catechol ring) of either the terminal unit or the extension unit or even the catechol rings at both the units. However, MS fragmentations of these two molecules do not allow distinguishing between two hypotheses concerning the structure of the oxidized catechol rings of PB2: does it correspond to a dienedioic acid structure, or is it a lactone [i.e. (5-Oxo-tetrahydro-furan-2-yl)-acetic acid group] as observed for

Table 2
The fragments obtained after MS and MS^2 analysis of each of the four major peaks from the HPLC profile.

Peak (product) name	Retention time (min)	UV lambda max (nm)	MS fragment $[M-H]^-$	MS^2 fragments
PB2-X	11.6	270	609	565, 457, 439
PB2-X_2	12.5	270	641	597, 623, 439, 319
PB2-X_3	18.1	270	609	565, 407, 319
PB2	23.6	280	577	559, 451, 425, 289

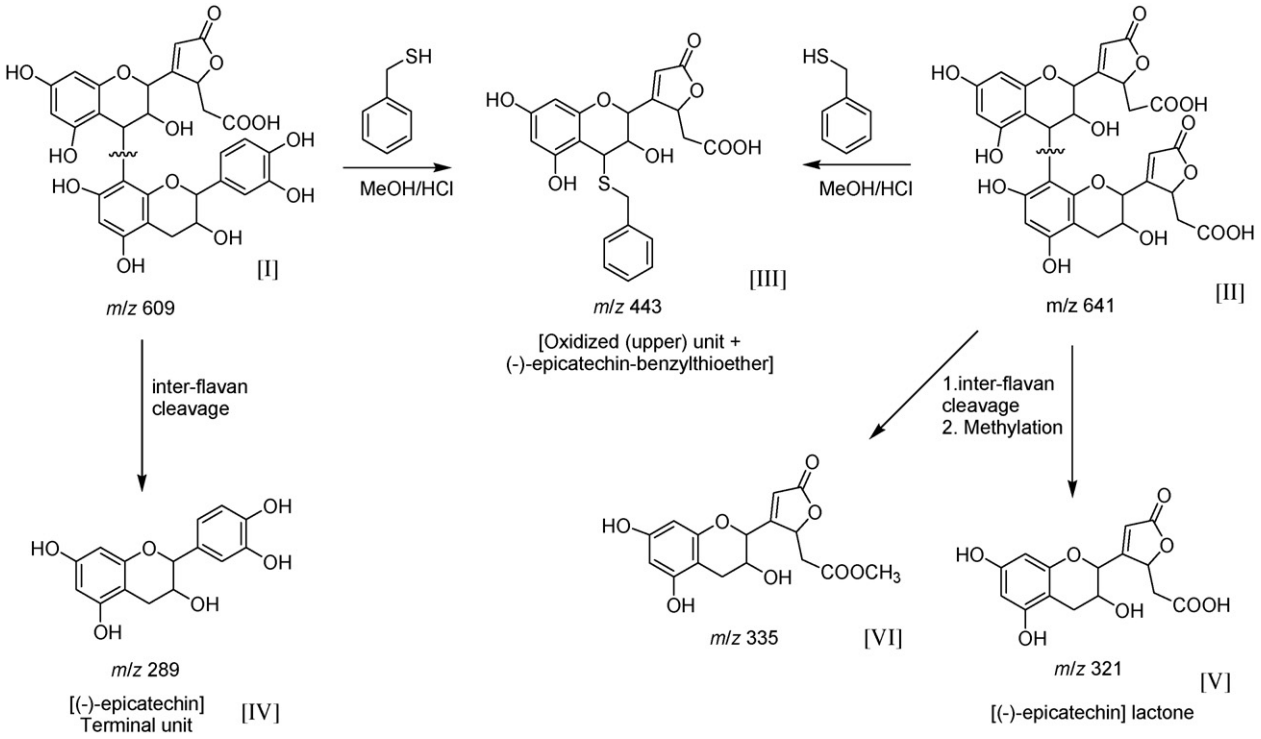


Scheme 3. Proposed MS fragmentation of PB2 dimer formed from thiolysis reaction.

PB2-X product [18]. Therefore, in order to complete the structural characterizations; PB2, PB2-X_2 & PB2-X_3 were subjected to thiolysis reaction and the products were analyzed by HPLC-UV-ESI-MS. The fragments obtained from multiple fragmentation mass spectrometry (MS^n) of the four main degraded products are illustrated in Table 2.

3.5. MS fragmentation study of the thiolysis reaction products of PB2, PB2-X_2 and PB2-X_3

The thiolysis reaction has been used frequently for the characterization of procyanidins [28]. The reaction occurs when procyanidins are heated in the presence of acid and benzyl mer-



Scheme 4. Proposed MS fragmentation of PB2-X_2 and PB2-X_3 (oxidized products) formed from thiolysis reaction.

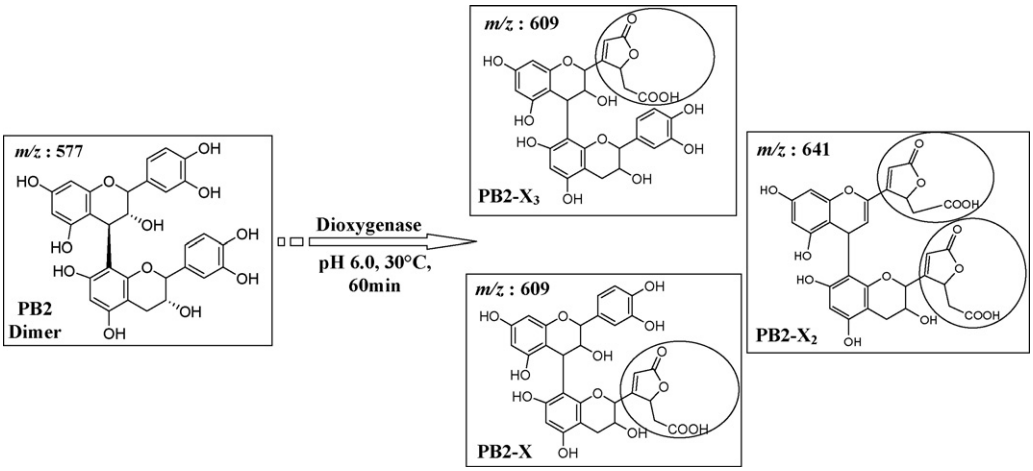


Fig. 5. The proposed dioxygenase reaction mechanism on PB2 & resulting oxidized products.

captan (also known as toluene-α-thiol, phenylmethane thiol) and corresponds to the acidic cleavage of the inter-flavan linkage of procyanidins. Terminal units are liberated in their free epicatechin form whereas extension units are liberated as a flavanyl carbocation immediately converted into the corresponding benzylthioether adduct [29]. In principle, this reaction permits determination of both chain length and composition of a procyanidin molecule when thiolysed products are analyzed by HPLC. Problems arise if the procyanidin molecule analysed is heterogeneous or if the thiolysis reaction is incomplete [30]. Freeze-dried samples of PB2, PB2-X_2 and PB2-X_3 were subjected to the thiolysis reaction as described by Guyot et al. [14]. The reaction products were then analyzed by HPLC coupled with UV-Visible and MS detection. As expected, analysis of the thiolysis products of PB2 revealed two reaction products [peak 1 (RT - 19.3), *m*/*z* 289 and peak 2 (RT - 42.2), *m*/*z* 411, Fig. 4]. These two products were thus identified as (−)-epicatechin and (−)-epicatechin benzylthioether (Scheme 3).

When PB2-X_2 (*m*/*z* 641) & PB2-X_3 (*m*/*z* 609) were submitted to LC–MS after thiolytic reaction both of them resulted in getting a common peak at fragment at *m*/*z* 443, which is the oxidized extension unit (dioxygenase action) coupled with the benzylthioether. The three other fragments detected were, one at *m*/*z* 289, identified as the terminal unit released from pseudo-molecular ion *m*/*z* 609; second one *m*/*z* 321 and the third one at *m*/*z* 335 formed from the pseudo-molecular ion of *m*/*z* 641 (Scheme 4). In comparison with earlier works dealing with the oxidation of monomeric catechin [31] the fragment with *m*/*z* 335 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. Similarly (unmethylated) oxidation derivatives of epicatechin have also been structurally elucidated. Hence, the fragment with *m*/*z* 321 was identified to be the unmethylated oxidized epictaechin. Fan et al. [32] identified similar molecules termed viniferone B or C [31] or (−)-epicatechin lactone. It was therefore confirmed, following MS^n studies that the enzyme dioxygenase from *A. fumigatus* is capable of modifying the catechol ring of either the extension unit (upper moiety) or the terminal unit (lower moiety) or even both the units of PB2. Subsequently these results paved the way for elucidating the mechanism of dioxygenase catalytic action (Fig. 5).

4. Conclusions

The extracellular enzyme from *A. fumigatus* MC 8 was found to be able to biotransform the procyanidin dimer, procyanidin B2 [(−)-epicatechin-(4β-8)-(−)-epicatechin] resulting in various biotransformed products. The enzyme was identified as a dioxygenase. It was purified to homogeneity and the various degraded products obtained after its catalytic activity with the dimeric substrate PB2 were characterized by mass spectroscopy. The isoelectric point (pI) of the enzyme was determined by chromatofocusing and found to be 3.5. A sequential strategy to purify the enzyme involving anion-exchange chromatography followed by gel-filtration was devised and performed. The purified dioxygenase enzyme had a molecular weight of 117 kilo Daltons (kDa) in non-denaturing conditions. The SDS-PAGE analysis indicated that the purified protein was a trimer. The MS spectrum of the identified and characterized products showed ions at *m*/*z* 609 and 641 that corresponded to the products obtained by the opening of the catechol ring of either the terminal unit or the extension unit and the catechol rings of both the units of PB2 dimer *via* intra-diol dioxygenase cleavage. The pseudo-molecular ion of PB2 had *m*/*z* 577 in negative mode. Detailed MS^2 analysis following thiolysis experiments resulted in the structural elucidation of each of the biotransformed products. Subsequently, the mechanism of dioxygenase catalytic action was devised paving the way towards the novelty of the present work, as it is for the first time a dioxygenase reaction mechanism on a procyanidin dimer is being reported.

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