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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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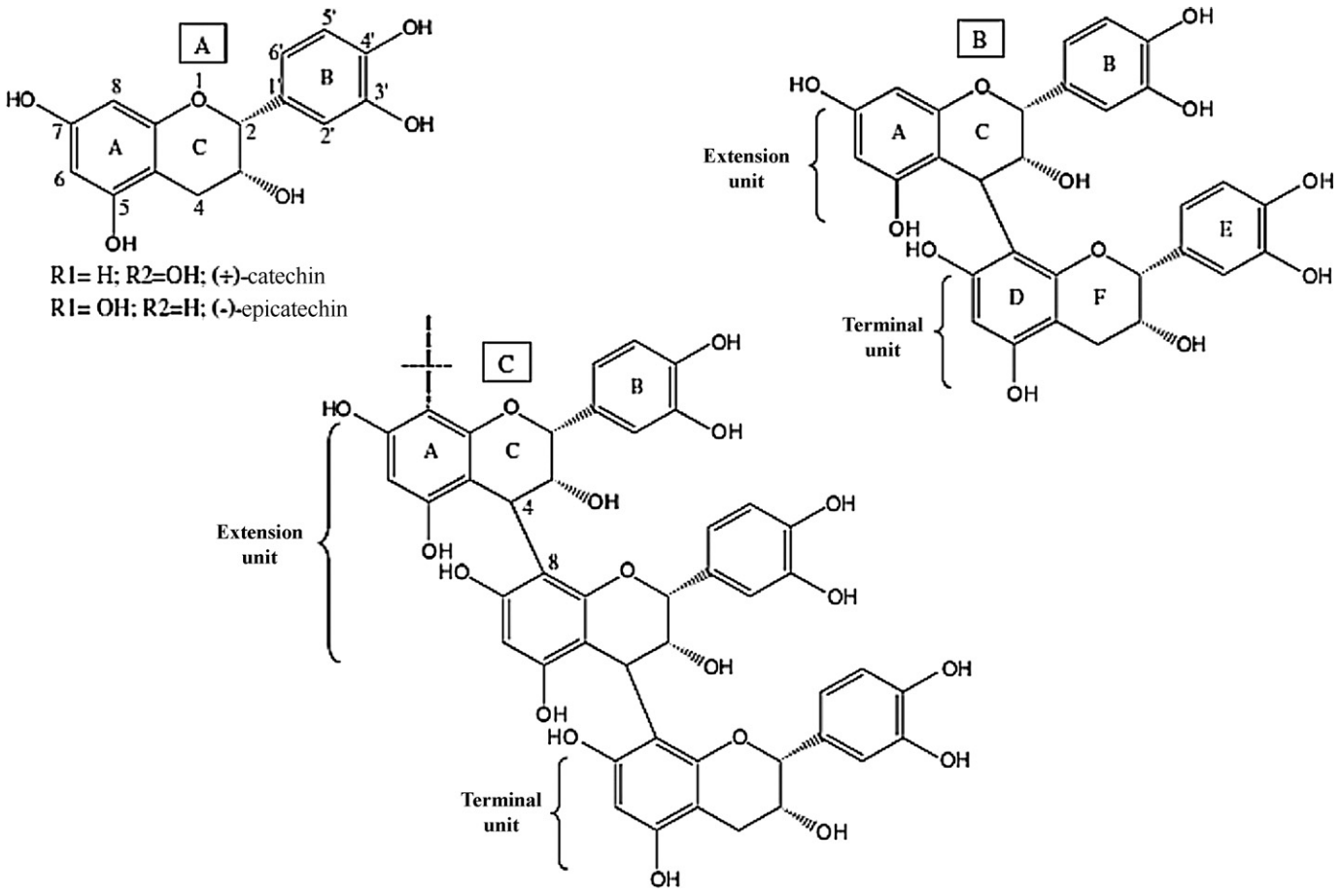

Fig. 1. General structures of procyanidins: (A) (+)-catechin and (−)-epicatechin monomers, (B) procyanidin B2 dimer, (C) proyanidin oligomers with C4-C8 linkage (*adapted from* Contreras-Domínguez et al., 2006).

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