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Carbon-14 biolabeling of flavanols and chlorogenic acids in *Crataegus monogyna* cell suspension cultures

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Abstract

Pure radiolabeled phenolic compounds (including catechins, procyanidins and chlorogenic acids) were obtained by administrating ^{14}C-L-phenylalanine to *Crataegus monogyna* cell suspension cultures. After a simple process of purification, pure compounds with high specific activities were recovered. In the present study, we describe a suitable method for obtaining labeled phenolics, which will be useful tools for in vivo feeding trials to trace their metabolic fate when consumed by animals and for in vitro activity mechanism studies.

Keywords: *Crataegus monogyna*; Cell suspension cultures; Radiolabeling; Catechins; Proanthocyanidins; Chlorogenic acids

1. Introduction

The disposal of radiolabeled phenolic compounds is very often required to assess their bioavailability from the diet, because the use of radioactive isotopes greatly facilitates the detection and positive identification of polyphenols present in trace amounts.

Among phenolic compounds, numerous studies concern flavan-3-ols and their oligomers, proanthocyanidins. They are some of the most abundant polyphenolic substances in the plant kingdom, and are an integral part of the human diet from fresh fruits, tea, chocolate and processed foods made from fruits (juices, wine, cider). Proanthocyanidins are known to possess a wide range of physiological activities. They are antioxidant, antimicrobial, anti-allergic, they act against caries, reduce blood pressure and inhibit some physiological enzymes and receptors (Chung, Yee Wong, Wei, Huang, & Lin, 1998; Cos et al., 2004; Elbøl Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005; Ricardo da Silva, Darmon, Fernandez, & Mitjavila, 1991; Santos-Buelga & Scalbert, 2000). However, relatively little is known about their bioavailability, the data on proanthocyanidin absorption and metabolism being sparse. Nevertheless, it seems that their absorption in the upper gastrointestinal tract is not high (Tsang et al., 2005) and that plasma does not contain proanthocyanidins or conjugates when fed to rats in a single meal (Donovan et al., 2002).

The aim of our work was to find a procedure that leads to obtain in an efficient way pure labeled proanthocyanidins which can be further applied to better characterize their bioavailability. The obtention of isotopically labeled proanthocyanidins by synthetic methods is often a complex multi-steps process that is tedious and leads to low yields, especially for the oligomers (Tulckmantel, Kozikowski, & Romanczyk, 1999). Alternatively, the use of biosynthetic methods can be suitable. Plant cell cultures have been studied extensively in order to produce plant secondary metabolites for use in medicine and the food industry. By adding a labeled precursor in the culture medium, labeled metabolites can be obtained.

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In order to obtain biolabeled proanthocyanidins, it is indispensable to find a source which produces high amounts of these molecules. *Crataegus* (Hawthorn) has long been used as a folk medicine and is widely utilized in pharmaceutical preparations mainly because of its neuro- and cardio-sedative actions and its low toxicity. Many of these properties are attributed to the presence of flavanols and proanthocyanidins. The proanthocyanidins occurring in *Crataegus* are primarily composed of (−)-epicatechin units, while the diastereoisomeric (+)-catechin is only present as a minor component (Rohr, Meier, & Sticher, 1999).

Besides accumulating proanthocyanidins, *Crataegus* cell suspensions have been shown to accumulate chlorogenic acids and other phenolic compounds (Bahorun, Trotin, & Vasseur, 1994). Chlorogenic acids (CGA) are a family of esters formed between certain *trans*-cinnamic acids and quinic acid, which are present in the human diet from coffee beverages, vegetables and fruits (Clifford, 1999). They have anti-bacterial, anti-mutagenic, anti-oxidant and many other biological activities (Nakamura et al., 1997; Rodriguez de Sotillo, Hadley, & Wolf-Hall, 1998). They can also regulate glucose metabolism by modulating glucose-6-phosphatase activity in vivo (Delcy, Rodriguez de Sotillo, Hadley, & Sotillo, 2006).

By using *Crataegus monogyna* cell suspension cultures, we report a procedure for the production of not only ^{14}C-labeled proanthocyanidins but also ^{14}C-labeled chlorogenic acids.

2. Materials and methods

2.1. Chemicals

L-[U-ring-^{14}C]-phenylalanine precursor (400 mCi/mmol) was purchased from Moravek (USA). All commercial chemicals used were of analytical grade quality, and solvents were purchased in HPLC grade quality.

2.2. Culture conditions and precursor feeding

Cell suspension cultures of *Crataegus monogyna,* established as described previously (Bahorun et al., 1994), were maintained under continuous fluorescent light (5000 lx) at 25 ± 1 °C in 250 mL Erlenmeyer flasks containing 50 mL of cell suspension on an orbital shaker (100 rpm). The maintenance medium (MM) contained macroelements, microelements, vitamins, 175 mM sucrose and 250 mg/L casein hydrolysate. It was supplemented with 10 μM of 2,4-dichloro-phenoxyacetic acid and 2.5 μM of kinetin. For experimental purposes, we inoculated a 7-day-old cell suspension into the medium at a 1/5 (v/v) ratio. After culturing cells (50 mL in 250 mL flasks), the labeled precursor (L-[U-ring-^{14}C]-phenylalanine, in ethanol–water (2:98, v/v) was fed at 5 μCi/mL half and half at two different times (days 3 and 4) with unlabeled L-phenylalanine to a final concentration of 0.5 mM. Cells were harvested on day 7 by vacuum filtration, rapidly washed with cold distilled water, weighed and stored at −20 °C until analysis.

2.3. Isolation and purification of labeled compounds

Frozen cells were homogenized with acetone–water (6:4, v/v), and the extract was concentrated in vacuo, dissolved in 20% aqueous methanol and chromatographed over a short cation-exchange resin column (Dowex®, 6 mm × 40 mm): after rinsing with water, the extract was eluted with 75% aqueous methanol, to yield a mixture of phenolic compounds. Pure compounds were isolated by semi-preparative HPLC (Varian, model 210) with a 4 × 250 mm Ultrasep RP18 column (4 μm) (Bischoff, Leonberg, Germany) at room temperature using the solvents water–trifluoroacetic acid 1% (100:0.5, v/v) (A) and methanol–trifluoroacetic acid 1% (100:0.5, v/v) (B) with the gradient system as follows: 20–40% B (0–40 min), 40–65% B (40–50 min), 65–100% B (50–51 min), 100% B (51–58 min). Detection was done at 286 and 306 nm, with a UV–Vis detector (Varian, model 345).

2.4. Identification of polyphenols and measurement of radioactivity

Unlabeled (+)-catechin, (−)-epicatechin, and procyanidin dimers B2, B4 and B5 were purified from *Crataegus* cell suspension cultures and unambiguously identified by NMR and mass spectrometry methods (Rohr, 1999). Radiolabeled compounds were identified by comparing their retention times to those of unlabeled molecules. The specific activities of the main polyphenols were determined using a Packard liquid scintillation analyzer after collection of each compound during semi-preparative HPLC purification. The liquid scintillation cocktail was Ultima Gold (Packard) and the counting efficiency was on average 96%. Counts were corrected for background (estimated at 20 cpm).

3. Results

3.1. Growth and polyphenols accumulation in Crataegus monogyna cultures

Two crucial aspects must be carefully established in biolabeling methods in suspension cultures: the optimal period for the precursor feeding and the suitable time when the culture must be stopped and cells be collected. To determine both, *Crataegus* cell suspension cultures were analyzed throughout a 10 day period (Fig. 1).

In previous studies for producing isotopically [^{14}C]-labeled polyphenols from *Vitis vinifera* cell cultures, we found that the rate of enrichment was higher when phenylalanine (PHE) was added at the beginning of the exponential phase compared to the middle or the end of this period (Vitrac et al., 2002). Moreover, two or three successive additions of this precursor led to a better rate of enrich-

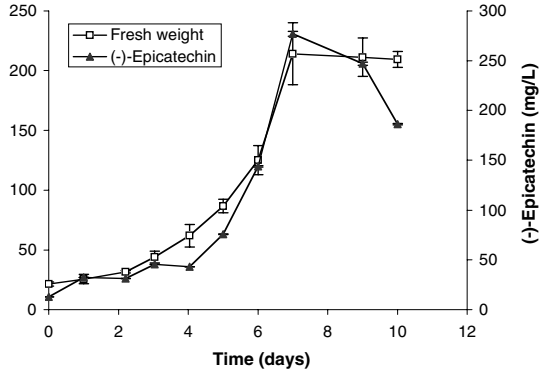

Fig. 1. The time courses of cell growth and (−)-epicatechin biosynthesis in *Crataegus monogyna* cell suspension cultures. Data are the mean ± SD of three replicates.

ment. We also found that a better incorporation was achieved when adding unlabeled PHE together with radiolabeled PHE. Indeed, unlabeled PHE can play the role of driving substance seeing that the very low concentration of added ^{14}C-PHE (12 μM) was similar to that of medium from cells cultured without added PHE. Thus, we decided to feed *Crataegus monogyna* cultures with ^{14}C-PHE at 5 μCi/mL half and half at two different times (days 3 and 4) together with unlabeled PHE to a final concentration of 0.5 mM.

Maximal production of (−)-epicatechin (about 300 mg/L) occurred on day 7, i.e. at the beginning of the stationary growth phase. Moreover, these suspension cultures showed good growth, about 200 g fresh wt/L. Therefore, we chose to apply these culture conditions to obtain maximal polyphenol production.

3.2. Specific activities of polyphenols

Polyphenols were extracted from cells harvested at day 7, purified on a cation-exchange resin (Dowex®) and isolated by semi-preparative HPLC. In order to identify these compounds, a similar extraction and purification was performed with a culture which had not been fed with the labeled precursor. The pure compounds obtained were analyzed by ^{1}H NMR and ESI-MS and their identity was confirmed by comparison with literature data (Rohr, 1999). Then, the radiolabeled polyphenols were assigned by comparison of their retention times with those from pure non-labeled compounds. A typical HPLC chromatogram of ^{14}C-labeled polyphenols is shown in Fig. 2.

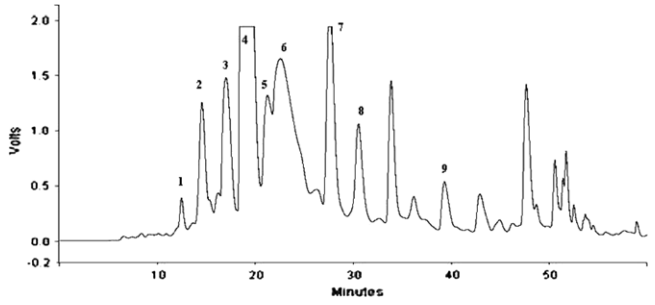

Fig. 2. Typical chromatogram of ^{14}C-labeled polyphenols recorded at 280 nm. **1** – procyanidin B4; **2** – (+)-catechin; **3** – procyanidin B2; **4** – 5-caffeoylquinic acid; **5** – mixture of a procyanidin trimer and a procyanidin tetramer; **6** – (−)-epicatechin; **7** – 5-*trans*-coumaroylquinic acid; **8** – 5-*cis*-coumaroylquinic acid; **9** – procyanidin B5.

Attribution of the position of the label in the produced compounds was based on knowledge of plant flavonoids biosynthesis pathways. Thus, since the phenyl ring of the L-[U-ring-^{14}C]-PHE is uniformely labeled, chlorogenic acids will be labeled at the aromatic ring, while catechins and procyanidins will be labeled at the B rings.

4. Discussion

Radioactivity was measured in order to calculate specific activities (Table 1). Overall, an average of 25% of the administered radioactivity was recovered in pure compounds, with 15% incorporation into proanthocyanidins and flavanols. These recovery values are higher than those found by Deprez, Mila, & Scalbert (1999), who recovered 3–5% of the total radioactivity in (+)-catechin and proanthocyanidin oligomers, when acetate or phenylalanine radioactive precursors were fed to willow tree cuttings. Our results are similar to those of Yousef et al. (2004), who obtained total recovery values of 14% or 15% for enriched flavonoid fractions from *Ohelo* suspension cultures. However, their values corresponded to whole fractions since they did not conduct to further purifications. Unlike the case with proanthocyanidins, only a few studies have been undertaken to produce biolabeled chlorogenic acids. Deprez et al. (1999) considered chlorogenic acid as a contaminant, but reported a recovery value up to 9%, a figure similar to that in the present study.

Specific activities for flavan-3-ol monomers and for procyanidin dimers were comprised between 680 and 1200 μCi/g and between 230 and 850 μCi/g, respectively. Deprez et al. (1999) obtained similar values for isolated (+)-catechin (543 μCi/g), procyanidin B3 (234 μCi/g) and trimer C2 (198 μCi/g), although higher amounts of

Table 1
Amounts and specific activities of polyphenols extracted from *Crataegus monogyna* cell suspension cultures

Compound	Amount (mg/L suspension culture)	Specific activity (mCi/mol pure compound)
(+)-Catechin	18	197
(−)-Epicatechin	308	333
Procyanidin B2	131	491
Procyanidin B4	24	132
Procyanidin B5	26	248
5-O-caffeoylquinic acid	280	708
5-O-*trans*-coumaroylquinic acid	64	294
5-O-*cis*-coumaroylquinic acid	37	1179

^{14}C-precursor were used (400 μCi). Nonetheless, our values for (−)-epicatechin and procyanidin B2, the main flavanols accumulated by *Crataegus monogyna* suspension cultures, are significantly higher, which represents a significant improve by comparison to those procedures. The present biolabeling method provides pure labeled molecules which are not commercially available and which cannot be easily obtained through synthetic routes. The use of *Crataegus monogyna* cell cultures led to high recovery values and high specific activities of pure proanthocyanidins and chlorogenic acids. Indeed, specific activities for all the compounds were higher than 100 μCi/mol, a value suitable for studying the intestinal absorption and tissue distribution of these compounds (Vitrac et al., 2003). Work on their rate of transepithelial transport and metabolism using human intestinal Caco-2 cells is now being carried out.

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