Food Chemistry 153 (2014) 130–133

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Phenylalanine ammonia-lyase, flavanone 3β-hydroxylase and flavonol synthase enzyme activity by a new *in vitro* assay method in berry fruits

Gema Flores *, Fernando De la Peña Moreno [1], Gracia Patricia Blanch, Maria Luisa Ruiz del Castillo

Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), Consejo Superior de Investigaciones Científicas (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 28 November 2013
Accepted 7 December 2013
Available online 12 December 2013

Keywords:
Phenylalanine ammonia-lyase
Flavanone 3β-hydroxylase
Flavonol synthase
Enzyme activity
Berry

ABSTRACT

An HPLC method for the determination of phenylalanine ammonia-lyase, flavanone 3β-hydroxylase and flavonol synthase enzyme activity is proposed. This method is based on the determination of the compounds produced and consumed on the enzymatic reaction in just one chromatographic analysis. Optimisation of the method considered kinetic studies to establish the incubation time to perform the assay. The method here described proved to be an interesting approach to measure the activities of the three enzymes simultaneously increasing the rapidity, selectivity and sensitivity over other exiting methods. The enzyme activity method developed was applied to strawberry, raspberry, blackberry, redcurrant and blackcurrant fruits.

1. Introduction

Flavonols are widely occurring phenolic compounds which exhibit multiple biological functions as a consequence of their powerful antioxidant properties (Vinson, Hao, Xuehui, & Zubik, 1998). Epidemiological and experimental studies indicate that flavonols can act as important proteasome inhibitors and apoptosis inductors (Chen et al., 2005). For this reason, long-term high consumption of flavonol-rich foods is associated with a lower risk of developing some diseases such as heart disease (Hertog, Feskens, & Kromhout, 1997), stroke (Keli, Hertog, Feskens, & Kromhout, 1996) and stomach cancer (Knekt et al., 1997). Although high contents of flavonols have been described in a number of vegetables (Sultana & Anwar, 2008), they are particularly abundant in edible berries (Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999).

Flavonols are produced in berries through the flavonoid biosynthetic pathway. Phenylalanine ammonia-lyase (PAL) enzyme catalyses the nonoxidative deamination of L-phenylalanine to yield cinnamic acid, a reaction that is generally considered to represent a key point at which carbon flux into this pathway is controlled. Cinnamic acid is subsequently converted to naringenin by the action of other enzymes. Later in the pathway three branch-point enzymes convert naringenin to flavones, dihydroflavonols and anthocyanidins, respectively. Specifically, the formation of dihydrokaempferol from naringenin is catalysed by flavanone 3β-hydroxylase (FHT) (Lukacin, Gröning, Schiltz, Britsch, & Matern, 2000). Dihydrokaempferol, similarly to the other dihydroflavonols, can be further oxidised to kaempferol by the action of flavonol synthase (FLS). PAL activity is associated with the accumulation of anthocyanins and other phenolic compounds in fruit tissues of several species, which is active during the development and ripening of fruits (Given, Venis, & Grierson, 1988). FHT and FLS are non-heme iron enzymes, dependent on Fe^{2+}, molecular oxygen, 2-oxoglutarate and ascorbate, the typical cofactors of the class of 2-oxoglutarate-dependent dioxygenases (Britsch, Heller, & Grisebach, 1981).

Enzyme activities have been extensively studied in plants using TLC and spectrophotometers to determinate the reaction products (Keli et al., 1996; Knekt et al., 1997; Sultana & Anwar, 2008). Kováčik and Klejdus (2012) reported the use of HPLC to determine PAL enzyme activity in two species, *Matricaria chamomilla* and *Arabidopsis thaliana*, and compared the results with a spectrophotometry assay. They stated that HPLC is a more accuracy method since it avoids interferences with cinnamic acid detection caused by other UV-absorbing compounds.

PAL activity has been assayed in grapes (Hrazdina, Parsons, & Mattick, 1984), apple (Faragher & Brohier, 1984), melon (Diallinas & Kanellis, 1994), sweet cherry (Wiersman & Wu, 1998) and strawberry (Cheng & Breen, 1991). FLS has been extracted from *Matthiola incana* (Spribille & Forkmann, 1984), *Petunia hybrida* (Forkmann, de Vlaming, Spribille, Wiering, & Schramm, 1986), *Dianthus caryophyllus*

* Corresponding author. Tel.: +34 91 562 29 00; fax: +34 91 564 48 53.
E-mail address: gemaflores@ictan.csic.es (G. Flores).
[1] Present address: Instituto de Investigaciones Biológicas (CIB-CSIC), Consejo Superior de Investigaciones Científicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.12.034

(Forkmann, 1991), *Citrus unshiu* (Moriguchi et al., 2002), grapes (Mattivi, Raffaele, Vrhovsek, Stefanini, & Velasco, 2006) and bilberry (Jaakola et al., 2002). However, to our knowledge, no information on PAL, FHT or FLS from raspberry, blackberry, red currant or blackcurrant can be found in the literature.

The goal of this investigation was to develop a HPLC method for the determination of the PAL, FHT and FLS enzyme activities assayed *in vitro* in one chromatographic analysis. The developed method was them applied to study the activity of PAL, FHT and FLS enzymes in strawberry, raspberry, blackberry, red currant and blackcurrant fruits.

2. Materials and methods

2.1. Chemicals and samples

HPLC grade methanol (MeOH) and acetonitrile (ACN) were supplied by Labscan Ltd. (Dublin, Ireland). Trifluoroacetic acid (TFA), polyclar AT, Tris/HCl, $FeSO_4$, Na-ascorbate, 2-oxoglutarate and ethylenediaminetetraacetic acid (EDTA), cinnamic acid, phenylalanine, naringenin, kaempferol and morin were purchased from Sigma (Steinheim, Germany). Ethyl acetate was provided by Scharlau. Milli-Q water was collected from a purification system (Millipore, Milford, MA, USA). Full-ripe strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus L.*), blackberry (*Rubus fructicosus L.*) and red currant (*Ribes rubrum*) fruits were acquired from the local supermarket whereas blackcurrant (*Ribes nigrum*) was grown under controlled conditions. The used varieties of the berries were Splendor (Egypt), Brilliance (Spain), Tupi (Spain), Rovada (Holland) and Hope (Scotland) for strawberry, raspberry, blackberry, redcurrant and blackcurrant, respectively. All the berries were picked up with uniform size, colour, ripeness and free from damage were utilised, as explained below.

2.2. Enzyme preparation

The enzyme preparation was basically the same for all fruits studied. The berries were first frozen at −80 °C and lyophilized. Subsequently, the crude enzymes were extracted as follows: the fruits were ground in a mill up to a fine powder. A total of 1.5 g of the powder obtained, 0.25 g of quartz sand, 0.25 g of Polyclar AT and 4 mL of 0.1 M Tris/HCl (containing 0.4% Na-ascorbate, pH 7.25) were properly homogenised (Halbwirth et al., 2009). The resulting homogenate was then centrifuged at 4 °C and 5000*g* for 10 min. Finally the supernatant was taken to accomplish PAL, FHT and FLS assays, as detailed below. This preparation was performed in triplicate for statistical studies.

2.3. Enzyme assays

Once obtained enzyme extract, the PAL, FHT and FLS activities were simultaneously measured as previously described by Halbwirth et al. (2009) with slightly modifications: 1.104 mmols of kaempferol, cinamic acid and phenylalanine, 2.208 mmols of naringenin, 50 μL of the enzyme preparation obtained above, 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 2.01 mM $FeSO_4{\cdot}7H_2O$, and 60 μL of a buffer made up of 0.1 M Tris/HCl + 0.4% Na-ascorbate (pH 7.25) were mixed. For both studies, the enzyme assay was then incubated for 45 min at 30 °C. After that, the assay was terminated by addition of 140 μL of ethyl acetate, 10 μL of acetic acid and 10 μL of 0.1 mM EDTA. The organic phase was finally taken to carry out the HPLC analysis as detailed below. In addition, the reaction in absence of enzyme preparation (so-called blank) was also performed for comparison.

2.4. HPLC analysis

Chromatographic analyses of the ethyl acetate phase were performed employing a Konik-Tech model 560 (Barcelona, Spain). Morin was used as internal standard. Liquid chromatograph fitted with a manual injection valve (model 7725i, Konik-Tech, Barcelona, Spain) having a 20-μL sample loop and an ultraviolet (UV) detector operated at 360 nm for morin and kaempferol, 280 nm for cinnamic acid and 290 nm for naringenin. The separation was accomplished on a ODS reverse phase (C18) column (250 × 4.6 mm i.d., 5-μm particle size, ACE, Madrid, Spain). The elution was performed by using solvent A (H_2O containing 0.1% TFA) and B (ACN) at a constant flow rate of 1.0 mL/min. A linear gradient was applied from the initial eluent composition (A:B, 75:25, v/v) up to A:B, 70:30 (v/v) for the first 15 min and then up to final composition (A:B, 65:35, v/v) within 25 min. Data acquisition was carried out by using Konikrom Plus (KNK-725-240). Identification of morin, cinnamic acid, naringenin and kaempferol was based on the comparison between the retention times obtained from the standards and from the chromatographic signals in the samples run under the same experimental conditions. Each sample was injected twice. For comparison, blanks were accomplished by measuring the content of cinnamic acid, naringenin and kaempferol in absence of enzyme extract. The LC equipment was carefully washed by passing methanol through the whole system for 15 min after every single run.

2.5. Calibration curves

Known volumes (1.5–32.5 μL) of a standard solution of 90 mg L^{-1} of cinnamic acid, naringenin and kaempferol were diluted up to 100 μL to make the calibration curves. The standard solution was prepared in 5 μL of 3.48 mM 2-oxoglutarate, 5 μL of 2.01 mM $FeSO_4{\cdot}7\ H_2O$, 60 μL of a buffer made up of 0.1 M Tris/HCl + 0.4% Na-ascorbate (pH 7.25), 140 μL of ethyl acetate, 10 μL of acetic acid and 10 μL of 0.1 mM EDTA to simulate the enzyme assay. A 12.5 μL-volume of a solution of 90 mg/L of morin (equivalent to 160 ng) were used as the internal standard. Duplicate injections of 20 μL of the ethyl acetate phase containing the target compounds were sampled into HPLC. Calibration curves were obtained for cinnamic acid, naringenin and kaempferol by representing the injected amount (ng) of each compound versus the relative peak area obtained (absolute area of the analyte/area of morin).

2.6. Statistical analyses

Data are expressed as mean values ± 95 % confidence interval. Analysis of variance (ANOVA) was performed by one way analysis of variance with significant differences between means determined by the Student́s *t*-test. The values used were always the mean of three replicates performed. JMP Statistics software package version 8 was used for statistical analyses (SAS Institute Inc., NC).

3. Results and discussion

A new method for the determination of the PAL, FHT and FLS enzyme activities by HPLC is proposed. It is based on the separation and quantification of the compounds produced and consumed from the enzymatic assays. The method developed was applied to strawberry, raspberry, blackberry, red currant and blackcurrant fruits.

First, the chromatographic conditions were optimised. As an example, Fig. 1 represents the chromatogram obtained from the HPLC analysis of a standard solution (50 μg mL^{-1}) of morin,