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Simulated digestion of proanthocyanidins in grape skin and seed extracts and the effects of digestion on the angiotensin I-converting enzyme (ACE) inhibitory activity

Katherina Fernández *, Javiera Labra

Bioengineering Laboratory, Department of Chemical Engineering, Faculty of Engineering, Universidad de Concepción, Barrio Universitario s/n, Concepción, Chile

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ABSTRACT

This study investigated the effect of *in vitro* gastrointestinal digestion on the stability and composition of flavan-3-ols from red grape skin and seed extracts (raw and purified, which are high in proanthocyanidins (PAs)). In addition, the effects of digestion on the angiotensin I-converting enzyme (ACE) inhibitory activities of these extracts were evaluated. The extracts were digested with a mixture of pepsin-HCl for 2 h, followed by a 2 h incubation with pancreatin and bile salts including a cellulose dialysis tubing (molecular weight cut-off 12 kDa) at 37 °C with shaking in the dark and under N_2. Under gastric conditions, the mean degree of polymerisation (mDP) of seed extracts, raw (mDP ≈ 6, $p < 0.05$), and purified (mDP ≈ 10, $p < 0.05$) was stable. The mDP of the raw skin extracts increased from 19 to 25 towards the end of the digestion. The PAs were significantly degraded (up to 80%) during the pancreatic digestion, yielding low-molecular-weight compounds that diffused into the serum-available fraction (mDP ≈ 2). The overall mass transfer coefficient (K) of the seed extracts was 10^{-7} m^2/s. After simulated gastrointestinal digestion, over 80% of ACE inhibition by raw seed and skin extracts was preserved. However, the purified seed and skin extracts lost their ability to inhibit ACE after intestinal digestion.

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1. Introduction

Flavanol compounds are monomers and oligomers of flavan-3-ols, and are identified as catechins and proanthocyanidins (PAs, condensed tannins), respectively (Hummer & Schreier, 2008). In grapes, several PAs have been characterised in the literature (Chira, Schumauch, Saucier, Fabre, & Teissedre, 2009; Kennedy & Jones, 2001; Monagas, Gomez-Cordoves, Bartolome, Laureano, & Da Silva, 2003); they mainly consist of catechin and epicatechin and their galloylated derivatives, and their composition differs between seed and skin.

Grape seed and skin extracts have been shown to have a high potential to protect against several diseases (Rasmussen, Frederiksen, Krogholm, & Poulsen, 2005; Yang, Kim, Yang, Kim, & Kwon, 2012). Epidemiological and biological evidence suggest that dietary flavan-3-ols have a health-protective role, including cancer (Neuhouser, 2004), cardiovascular disorders (Ding, Hutfless, Ding, & Girotra, 2006), obesity, and diabetes (Thielecke & Boschmann, 2009), as well as neurodegenerative disorders (Ksiezak-Reding et al., 2012). This evidence has led to increased interest in the bioavailability of these compounds from foods. Flavan-3-ol bioavailability depends on numerous factors, including source and type of flavan-3-ol, digestive release, absorption, metabolism, and elimination (Feng, 2006).

Determining the true bioavailability of any class of phytochemicals requires the collection of data concerning the absorption, metabolism, tissue and organ distribution, and excretion of these compounds. Performing such studies in animals or human subjects is complex and expensive and raises moral and ethical questions (McDougall, Fyffe, Dobson, & Stewart, 2005).

In vitro methods are useful to study the stability of compounds under gastrointestinal conditions and their release from food matrices. Various studies have investigated the effect of *in vitro* gastrointestinal digestion on the stability of phenolic compounds from wine, and these studies revealed that the antioxidant activity after digestion was greater than that of the original samples, as the result of the release of simple phenols (caffeic acid, *p*-coumaric acid, and protocatechualdehyde) (Noguer et al., 2008). In addition, quantitative changes, particularly for phenolic acids, were observed as the result of the resveratrol and chlorogenic acid, the release of caffeic acid and the formation of other derivatives with high antioxidant activities (Gumienna, Lasik, & Czarnecki, 2011). Anthocyanins from red wine were found to be stable under gastric

* Corresponding author. Tel.: +56 41 220 4534; fax: +56 41 224 3750.
E-mail address: kfernandeze@udec.cl (K. Fernández).

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conditions; however, after pancreatic digestion, the total anthocyanins were poorly recovered from the serum- and colon-available fractions (McDougall et al., 2005). Only one report regarding the simulated gastrointestinal digestion of *Vitis vinifera* seed powder was found in the literature. In that study, polyphenols (catechin, epicatechin, and gallic acid procyanidins B1, B2, C1, and EB5) were released during digestion. These compounds had good scavenging potential for superoxide radicals, hydroxyl radicals, and singlet oxygen (Janisch, Olschlager, Treutter, & Elstner, 2006). No reports on skin grape extracts were found in the literature.

It is interesting to note that after the simulated digestion of wines or grape seed extracts, the antioxidant capacity was the primary bioactivity studied, even though grape extracts have other known bioactivities, such as the *in vitro* inhibition of angiotensin I-converting enzyme (ACE) activity by seed and skin extracts from *País* grapes, which are high in PAs. (Eriz, Sanhueza, Roeckel, & Fernandez, 2011; Godoy, Roeckel, & Fernandez, 2012). The effect of those extracts on ACE was related to the type of extract, structural features and molecular size.

ACE is a metallo-glycoprotein linked to the membrane that catalyses the hydrolysis of the decapeptide angiotensin I by cleaving off the C-terminal dipeptide, producing the octapeptide angiotensin II, which is responsible for increasing blood pressure. The inhibition of ACE activity is a therapeutic approach for the treatment of hypertension and associated coronary diseases in humans (Loizzo et al., 2007).

The bioavailability of PAs depends on their chemical characteristics. It has been suggested that only PA dimers and trimers can be absorbed *in vivo* (Deprez, Mila, Huneau, Tome, & Scalbert, 2001) and that polymeric PAs are not absorbed but can be degraded into various aromatic acids by the microflora (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Thus, the compounds are absorbed through the gut barrier and their absorption depends on their mean degree of polymerisation (mDP). Nevertheless, the *in vitro* inhibition of ACE by skin and seed PAs was highly effective when the mDP of the PA chain was high (Eriz et al., 2011).

In this study, grape skin and seed extracts (raw and purified, high in PAs) were submitted to *in vitro* simulated gastrointestinal digestion to investigate the degradation of these compounds under conditions close to physiological conditions and to evaluate the effect of digestion on the inhibition of ACE.

2. Materials and methods

2.1. Materials

The acetone, *n*-hexane and ethanol used in the PA extraction and purification were obtained from Merck (Darmstadt, Germany), with 98% purity. Water was purified in the laboratory using a Millipore system (Millipore, Milli-Q plus, Bedford, MA). The HPLC-grade acetic acid, acetonitrile and trifluoroacetic acid (TFA) used in the phloroglucinol catalysis were obtained from Merck, with 98% purity. Hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), angiotensin I-converting enzyme (ACE) from rabbit lung (A6778), phloroglucinol, (+)-catechin (C), pancreatin and bile salts were obtained from Sigma–Aldrich, (St. Louis, MO). Toyopearl HW 40F chromatography resin was purchased from Supelco (Bellefonte, PA).

2.2. Proanthocyanidin extraction and purification from grapes

País grapes were obtained from Quillón Valley, Bio Bio Region, Chile, on March 18, 2011 (in *verason*). The PA extraction was based on Kennedy and Jones (2001) with the modifications used by Villarroel (2009). The skin and seeds of 200 grapes were manually separated and extracted in Erlenmeyer flasks, using 250 ml of acetone/water solution (2:1, v/v). The extraction was carried out in an orbital shaker at 60 rpm with continuous agitation (G24 Environmental Incubator Shaker; New Brunswick Scientific Co., Inc., Edison, NJ) in the dark for 15 h at room temperature. N_2 was bubbled through the sample to minimise oxidation. Then, the extracts were filtered to separate the soluble compounds from the waste solid, and the solution was concentrated to 50 ml at reduced pressure and temperature (<35 °C) in a rotary evaporator (Rotary Evaporator, Bibby RE 100, Stone, UK) to remove the acetone. The seed extract solutions were washed three times with 50 ml of *n*-hexane in a separating funnel to remove lipid-soluble compounds. Finally, both aqueous extracts were centrifuged at 8873*g* for 20 min, filtered, and frozen at −18 °C prior to further characterisation. These extracts are referred to as raw extracts.

The raw extracts were separately purified using size exclusion chromatography with Toyopearl® HW-40F resin (414 ml) packed in an Omnifit® column (Cambridge, UK) of 50 cm length × 3.5 cm id, 7 ml/min. The column was equilibrated with ethanol/water (55:45, v/v; two column volumes). The skin and seed extracts were separately loaded onto the column, and the sugars and phenolic acids were removed with ethanol/water (55:45, v/v; three column volumes). This fraction was discarded and not analysed. Then, acetone:water (60:40, v/v; one column volume) was used to elute the PA fraction. The acetone present in the PA fraction was evaporated at reduced pressure and temperature (<35 °C), and the extracts were stored at 4 °C prior to further analysis. These extracts are referred to as purified extracts.

2.3. Gastrointestinal digestion of grape extracts

The *in vitro* gastrointestinal digestion procedure was performed with both the raw and purified extracts. The procedure was adapted from a published method (Gil-Izquierdo, Zafrilla, & Tomás-Barberán, 2002) that showed a significant correlation between the *in vitro* and *in vivo* measurements of iron bioavailability. The method consists of two sequential steps: an initial pepsin/HCl digestion for 2 h at 37 °C to simulate gastric conditions, followed by digestion with bile salts and pancreatin for 2 h at 37 °C to simulate the conditions in the small intestine. The 2-h duration represents the average duration of gastrointestinal transit. The seed and skin extracts (raw and purified) (300 ml) were adjusted to pH 1.7 with 5 M HCl, and pepsin was added at 315 U/ml. The samples were then incubated at 37 °C in water for 2 h with shaking at 100 rpm. Every 30 min, aliquots (5 ml) of the post-gastric digestion were removed and frozen for further determination of PA and ACE inhibition in raw and purified extracts. The remainder of the sample was placed in a 500-ml glass beaker, and 4.5 ml of 4 mg/ml pancreatin and 25 mg/ml bile salts were added. The sample was then incubated for 2 h at 37 °C. A segment of cellulose dialysis tubing (molecular weight cut-off, 12 kDa) containing sufficient $NaHCO_3$ to neutralise the sample's titratable acidity was added, and the beaker was sealed with Parafilm. Every 30 min, aliquots (5 ml) of the solution outside the dialysis tubing were taken, and these OUT samples represent the material that remained in the gastrointestinal tract (colon-available fraction). Aliquots of the solution that had diffused into the dialysis tubing were also taken as the IN samples (serum-available fraction). The diffusion of $NaHCO_3$ out of the dialysis tubing represents the simplest and most convenient means to mimic the gradual increase in pH that occurs as the stomach contents enter the small intestine. All samples were heated to 90 °C for 1 min to terminate the digestion. Controls without added enzymes were run in parallel to differentiate the effects due to the presence of

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