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Inhibition of the angiotensin-converting enzyme by grape seed and skin proanthocyanidins extracted from *Vitis vinífera* L. *cv. País*

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ABSTRACT

The influence of two extracts of grape skin and seeds from *Vitis vinífera* L. cv. País (Chilean black grapes), rich in proanthocyanidins (PAs), was evaluated on the inhibition of the angiotensin I-converting enzyme (ACE), and the inhibition was related to the type and number of subunits of the polymeric PAs chain. Size exclusion chromatography was used to purify the extract and its characterization was made by acid catalysis depolymerization followed by HPLC. ACE activity was measured by quantitative HPLC, measuring the hyppuric acid (HA) produced from the hydrolysis of hippuryl-L-histidyl-L-leucine (HHL) by ACE. Structural compositions differed significantly between both extracts: the skin extracts do not exhibit epicatechin (EC) and epicatechin gallate (ECG), and the seed extracts did not present epigallocatechin (EGC). Skin extracts have a higher mean degree of polymerization (mDP) than seed extracts and a higher inhibition power (IC_{50} = 0.14 ± 0.03 μM and IC_{50} = 0.480 ± 0.03 μmol/L), respectively. The catechin (IC_{50} = 1495 ± 90 μmol/L) and epicatechin (IC_{50} = 1772 ± 121 μmol/L) monomers exerted lower inhibition than the either grape extract. The structural differences between grape skin and seed PAs could influence the ACE inhibition capacity. The larger inhibitory power of skin extract was associated to greater OH availability, higher mDP and the presence of EGC.

1. Introduction

The flavanol compounds are monomers and oligomers of flavan-3-ols, and are identified as catechins and proanthocyanidins (PAs, condensed tannins), respectively (Hummer & Schreier, 2008). The flavan-3-ols type that contains the PA chain depends on the natural source from which they are extracted. In grapes, several PAs have been characterized in the literature (Chira, Schumauch, Saucier, Fabre, & Teissedre, 2009; Kennedy & Jones, 2001; Monagas et al., 2003), finding that the PAs mainly consist in catechin and epicatechin and their derived galloylated. The PA structures are characterized by multiple substitutions of hydroxyl groups, which are directly related with its reactivity (Pietta, 2000). Several biological activities have been associated to the PAs, including: formation of tannin–protein complexes, formation of chelates with metal ion, and action as reducing agents (Aron & Kennedy, 2008; Noguer et al., 2008). Thus, one would expect that grape skin and seed extracts, rich in PAs, should present different abilities to interact with macromolecules, including enzymes. Indeed, it has been reported that skin and seed PAs differ significantly in composition and in their mean degree of polymerization (mDP). Skin PAs have a higher mDP and a lower proportion of galloylated units than seed PAs (Vidal et al., 2003).

A natural source with potential to produce PA extracts is *Vitis vinífera* L. cv. País, which is an ancestral Chilean grape variety introduced by Spanish monks during the XVI century. This variety is still cultivated in Chile, although it is not a priority for wine producers because it results in low quality wine characterized by its roughness and unbalance (Barría, 2007) These undesirable wine characteristics indicate the existence of a large amount of phenols, which is a great opportunity for the bioprocessing industry to use these undervalued products in the creation of other valuable products.

The angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is a highly glycosylated zinc dipeptidyl-carboxypeptidase that plays an important role in the rennin-angiotensin system (RAS), where the latter regulates the arterial blood pressure and the electrolyte balance in mammals (Velez, 2009). ACE catalyzes the degradation of angiotensin I to angiotensin II, a potent vasoconstrictor, by removing the carboxyl terminal dipeptide, His–Leu (Skeggs, Kahn, & Shumway, 1956). ACE also catalyzes the degradation of the vasodilator Bradykinin (Ondetti, Rubin, & Cushman, 1977). The inhibition of ACE activity is a “therapeutic approach” for the treatment of hypertension and associated coronary diseases in humans (Loizzo et al., 2007).

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Currently, several synthetic drugs that act as ACE inhibitors have been synthesized and are used to treat arterial hypertension in humans, such as *Captopril* and *Enalapril*. Nevertheless,the identification of natural sources that act as ACE inhibitors had also been reported. The descriptions of peptides as ACE inhibitors have mainly focused on milk (FitzGerald, Murray, & Walsh, 2004), vegetable and other animal proteins (Li, Le, Shi, & Shrestha, 2004), although there are other molecules that can effect ACE inhibition.

Today, epidemiological studies have associated the consumption of flavonoid-rich foods with reduced risk of cardiovascular diseases and decreased blood pressure in humans (Hollman, 2001; Kris-Etherton & Keen, 2002). However, only few studies have shown that purified PAs inhibit ACE activity *in vitro* (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003). The main problem with these results is the lack of knowledge about the source and the structural characteristics of the oligomeric PAs used; both are very relevant aspects in determining the reactivity of those towards ACE. The metabolic fate of proanthocyanidins is still elusive as there is conflicting evidence on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols in humans and animals (Prasain et al., 2009; Serrano, Puupponen-Pimia, Dauer, Aura, & Saura-Calixto, 2009). A first approach in the development of a natural hypothensive extracts considers to evaluate the reactivity of natural extracts from País grapes on ACE *in vitro*.

The aim of this study was to evaluate the influence of two types of PA-rich extracts produced from grape skin and seed of *Vitis vinífera* L. cv. País, harvested from the Itata Valley, Bio–Bio region, Chile, on the inhibition of ACE activity, and to relate the concentration, type and number of subunits composing the polymeric PAs chain with the inhibitory power. This study also evaluates the PA content and structural characteristics of the País grapes seed and skin, variables not previously assessed.

2. Materials and methods

2.1. Reagents and standards

The (+)-catechin (C), (−)-epicatechin (EC), hippuryl-histidyl-leucine (HHL), hyppuric acid (HA), purified angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) from rabbit lung, phloroglucinol, ascorbic acid and Tris buffer were purchased from Sigma Aldrich (St. Louis, MO). Chromatographic grade acetone, acetonitrile, acetic acid, n-hexane, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Solvents of analytical grade methanol, ethanol and 37% hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). The bidistilled water used in all solutions was purified to HPLC grade using a Millipore Milli-Q as a fast water system (Bedford, MA, USA). Toyopearl HW-40F column packing material was obtained from Supelco (St Louis, MO, USA).

2.2. Proanthocyanidins isolation from grapes

The grapes were harvested a month before the commercial ripening from three locations (Guarilihue, Ñipas and Quillón) in the Itata Valley, Bio Bio Region, Chile. The skins and seeds of 200 grapes were manually separated and extracted in Erlenmeyer flasks using 250 mL of acetone and water (2:1, v/v) for 15 h at 35 °C and 250 rpm, in absence of light to reduce oxidation (Kennedy & Jones, 2001). The skin and seed extracts were separately filtered and concentrated under reduced pressure and temperature (<35 °C) to remove the acetone. Seed extracts were washed 3 times with 50 mL of n-hexane in a separator funnel to remove liposoluble compounds. Finally, both extracts were centrifuged at 2218×*g* for 5 min, filtered, and frozen at −18 °C for further purification.

2.3. Purification and characterization of proanthocyanidins

The raw extracts obtained in step 2.2 were purified according to size exclusion chromatography using Toyopearl® HW-40F resin packed in an Omnifit column (420 × 35 mm, 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 into the column, the sugars and phenolic acids were removed with ethanol: water (55:45, v/v) (three column volumes). This fraction was discarded and not analyzed. Then, acetone: water (60:40, v/v) (one column volume) was used to elute the proanthocyanidins fraction. The acetone present in proanthocyanidins fraction was evaporated at reduced pressure and temperature (<35 °C) and the extracts was lyophilized separately to yield 2.57 mg/g grape of seed and 1.11 mg/g grape of skin, which were stored at 4 °C for further analysis.

The PAs of each extract were characterized by an acid-catalyzed depolymerization method followed by HPLC detection (Cerpa-Calderon & Kennedy, 2008; Kennedy & Taylor, 2003). A solution of 0.1 mol equi./L HCl in methanol, containing 50 g/L of phloroglucinol was reacted with the seed and skin extracts (5 g/L) at 50 °C for 20 min. Then, the mixture was combined with 5 volumes of aqueous sodium acetate 40 mmol/L, to stop the reaction. The procedure was performed in duplicate for all tested samples. The compounds were detected with an HPLC Merck-Hitachi chromatograph LaChrom L7000 Series, with a gradient pump L-7100, Autosampler L-7200, UV detector L-4250 (wavelength 280 nm) and two Chromolith® Performance Series RP-18e columns (Merck, Darmstadt, Germany). The mobile phase consisted in Milli-Q water with 1 v/v aqueous acetic acid (mobile phase A) and acetonitrile with 1 v/v acetic acid (mobile phase B) and elution was performed with 3% B for 4 min. The linear gradients used were: 3%–18% B for 14 min and 80% B for 2 min at a flow rate of 3 mL/min and 30 °C. The column was washed with 3% B for 2 min before performing the next injection. To quantify the samples, an external standard of catechin (100 mg of C/L) was used. The extract characterization gave values of mDP, the proportion of their components (C, EC, (—)-epigallocatechin (EGC), (—)-epicatechin gallate (ECG), (—)-epicatechin-phloroglucinol (EC–P), (—)-epicatechin gallate phloroglucinol (ECG–P), (—) epigallocatechin- phloroglucinol (EGC–P)), yield (>80 g/100 g) and concentration.

2.4. Determination of purified ACE activity

The ACE enzyme activity was determined by monitoring the hydrolysis of HHL to produce HA, which was separated and quantified by HPLC. The HPLC equipment was the same as previously described. The methodologies of Actis-Goretta et al. (2003) and Wu, Aluko, and Muir (2002) were compared to identify the analytical methodology with the best accuracy and lowest cost. Finally, the detection was performed at 228 nm and the mobile phase consisted of 0.1 v/v TFA in a Milli-Q/Acetonitrile (75:25, v/v) solution using a flow of 1 mL/min, with a retention time of 1.41 min for HA. The column was a LiChroCART® stainless steel (125 × 3 mm) unit packed with a 5 μm diameter stationary phase Purospher® STAR RP-18 (Merck, Darmstadt, Germany).

2.5. Inhibition of purified ACE by PAs from grape seed and skin extracts

An aliquot of 60 μL of HHL (10.8 mmol/L) and a combination of 10 μL of purified ACE (35.8 U/mL) mixed with 60 μL of grape extract (skin or seed) (0–1 mg/mL) were pre-incubated in parallel for 10 min in a water bath at 37 °C. The aliquots were fresh prepared using HCl-Tris 50 mmol/L buffer, 300 mmol/L NaCl, pH 8.3. The