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Influence of the structure and composition of the *País* grape proanthocyanidins on the inhibition of angiotensin I-converting enzyme (ACE)

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ABSTRACT

The influence of polymerisation (number of subunits), galloylation (content of esterified gallates) and type of subunit in the polymeric chain of proanthocyanidins (PAs) in skin and seed extracts of *Vitis vinífera* L. cv. *País* on the inhibition of angiotensin I-converting enzyme (ACE) was evaluated. Gel permeation chromatography was used to purify the extracts, which were characterised by acid-catalysis depolymerisation followed by HPLC. ACE activity was tested by quantitative HPLC, measuring the hippuric acid (HA) produced from hippuryl-L-histidyl-L-leucine (HHL) hydrolysis by ACE. The inhibitory activity on ACE was verified for all the obtained fractions, although the PAs' molecular size and structural composition affected the ACE inhibition. The higher inhibitory power of skin extract was associated with greater –OH availability, higher mean degree of polymerisation (*m*DP) and the presence of epicatechin-gallate (ECG) expressed as percentage of galloylation (%G). However, comparing only the structural composition (for samples with similar mDP), % epicatechin (EC) and ECG, <3 %G appeared to be the most important characteristic when considering inhibitory effect of both tissues. Thus, these results indicate that the ACE inhibition by grape PAs depend on the stereochemical configuration of the studied flavanols.

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1. Introduction

País grape is a variety commonly found in the Bio Bio Region, southern Chile. It is the oldest grape variety cultivated in Chile and is strongly resistant to plagues and soil adversities. In spite of this, it is slowly disappearing from the vineyards because the produced wines are not commercially attractive (Costa Barros, 2004). Previous works developed in our laboratory showed that the *País* grapes from the Itata Valley, Chile have high amounts of proanthocyanidins (PAs) when compared to other varieties, such as Carmenère or Pinot Noir (Villarroel, 2009). This fact suggests an opportunity to increase its market value.

The PAs, located mainly in grape skin and seeds, are highly complex phenolic compounds with molecular weights of over 500 (de Freitas & Mateus, 2001). These polymers are part of the flavanol family and are composed of flavan-3-ol subunits connected by C_4–C_8 or C_4–C_6 bonds. PA subunits are differentiated by their substitutions and the stereochemistry of their structures. The most common monomers are (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin-gallate (ECG) and (−)-epigallocatechin (EGC). Their bioactive properties have been reported to be determined by molecular composition and size (Bindon, Smith, Holt, & Kennedy, 2010), where the amount of hydroxyl groups determines PA reactivity towards the proteins (Acuie-Beghin, Sausse, Meudec, Cheynier, & Douillard, 2008; de Freitas & Mateus, 2001; Hagerman & Butler, 1981; Richard, Lefeuvre, Descendit, Quideau, & Monti, 2006; Siebert, 1999b; Siebert & Lynn, 1998; Siebert, Troukhanova, & Lynn, 1996), metallic ions (Foo, Lu, McNabb, Waghorn, & Ulyatt, 1997), polysaccharides (Bindon et al., 2010), etc. In addition, there are reports on the influence of the molecular size on the bioactivity of these compounds, indicating that the molecule is more active when is larger (Actis-Goretta, Ottaviani, & Fraga, 2006; Lotito et al., 2000; Eriz, Sanhueza, Roeckel, & Fernández, 2011). These factors show the need to determine the composition and to separate the different sizes of the PAs in order to understand their properties.

One bioactivity reported for the PAs is its inhibitory effect on angiotensin I-converting enzyme, ACE (Actis-Goretta, Ottaviani, Keen, & Fraga, 2003; Actis-Goretta et al., 2006; Eriz et al., 2011). This enzyme is a metallo-glycoprotein linked to the membrane that catalyses the hydrolysis of the decapeptide angiotensin I by cleavage of its C-terminal dipeptide, producing the octapeptide angiotensin II, which is responsible for increasing blood pressure. When this high blood pressure state is constant, the person affected is considered to be hypertensive. Hypertension can slowly damage kidney, veins, and heart, and thus should be controlled to avoid complications (Fernández, 1995). Even when there are effective medicines for that purpose, they have side effects such as coughing, headaches, nausea, and anxiety, which may affect patients' life

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quality (Atkinson & Robertson, 1979; Fernández, 1995). For this reason, it is interesting to study naturally-occurring substances that could have lower or no side effects and inhibit ACE.

At present, only one study analysed the influence of PA structure on the ACE inhibition which found that the degree of polymerisation defines the extent and specificity of ACE interaction (Ottaviani, Actis-Goretta, Villordo, & Fraga, 2006). However, the main problem with those results is the lack of knowledge of the PA source and structural characteristics (definition of PA's terminal and extension subunits). Thus, the influence of PA composition on ACE inhibition is unclear. This information is highly relevant when trying to determine the reactivity and inhibition mechanism, and to enhance its use to develop a natural medicine.

The main objective of this work was to evaluate the influence on ACE inhibition activity by the number of subunits (based on its mean degree of polymerisation, mDP) and the type of subunits (C, EC, ECG, EGC) of the PAs extracted from the seed and skin of *País* grapes. Thus, the PAs of different molecular sizes present in the seed and skin extracts were purified and the composition and size of each sample (fractions) were determined. Next, their inhibitory activity on ACE was compared, and the relationships between the fractions properties and their inhibitory ability were determined. Finally, the inhibitory power of PAs was compared with Enalapril, a chemical inhibitor of ACE.

2. Materials and methods

2.1. Chemicals

Acetone, *n*-hexane and ethanol used in PA extraction and purification were obtained from Merck, Germany, with 98% purity. Water was purified in the laboratory by a Millipore system (Millipore, Milli-Q plus, USA). Acetic acid, acetonitrile and trifluoroacetic acid (TFA) used in the phloroglucinol catalysis were obtained from Merck, Germany, with 98% purity, as HPLC grade. Hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL) and angiotensin I-converting enzyme (ACE) from rabbit-lung (A6778), phloroglucinol, (+)-catechin (C) and (−)-epicatechin (EC) were obtained from Sigma–Aldrich, USA. Enalapril Maleate LCH (10 mg) was obtained from Laboratorios Chile, Chile.

2.2. Sample collection and proanthocyanidin extraction

País grapes were obtained from Quillón Valley, Bio Bio Region, Chile on March 18, 2010 (in *verason*). The PAs extraction was based on Kennedy and Jones (2001), with modifications done by Villarroel (2009). 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). Extraction was carried out in an orbit shaker at 60 rpm with continuous agitation (New Brunswick Scientific Co., Inc., G24 Environmental Incubator Shaker, USA) in darkness, for 15 h at room temperature and bubbling N_2 to minimise compound oxidation. Then, the extracts were filtrated to separate soluble from waste solid and the solution was concentrated to 50 ml under reduced pressure and temperature (<35 °C) to remove the acetone in a rotary evaporator (Bibby, Rotary Evaporator, RE 100). Seed extracts solutions were washed three times with 50 ml of n-hexane in a separator funnel to remove liposoluble compounds. Finally, both aqueous extracts were centrifuged at 8873*g* for 20 min, filtered, and frozen at −18 °C for further purification and characterisation.

2.3. Proanthocyanidin fractionation and characterisation

Both extracts were separately purified according to size exclusion chromatography using a Toyopearl® HW-40F resin (414 ml) packed in an Omnifit column (50 cm length × 3.5 cm id, 7 ml/min). This step was carried out following the protocol described by Kennedy and Taylor (2003), changing methanol for ethanol and the volumes of the 5th and 6th solutions from 1 to 2 and 3 volumes, respectively. Five fractions from skins and five from seeds (F-A, F-B, F-C, F-D and F-E) were isolated, concentrated in a rotary evaporator to remove the organic solvent, and the aqueous phase was lyophilised to a dry powder for further analysis (Labconco, Freezer Dry System, USA). Mass recovery was 94.74% for the skin extract and 48.85% for the seed extract on d.w.

The amount of total phenols present in the extracts were determined by the Folin–Ciocalteau method (Singleton, Orthofer, & Lamuela-Raventos, 1999) and the composition of each fraction was determined by acid-catalysis in the presence of excess phloroglucinol based on the protocol described by Cerpa-Calderon and Kennedy (2008). Phloroglucinol adducts were analysed by a reverse HPLC technique as described by Kennedy and Taylor (2003), using two Chromolith® Performance Series RP-18e columns (100 × 4.6 mm) connected in series and protected by a precolumn Chromolith® RT 18 (5 × 4.6 mm) (Merck, Darmstadt, Germany). The composition, mean degree of polymerisation (mDP), mean molecular weight (mMW), yield percentage (%Y), percentage of galloylation (%G) and percentage of prodelphidin units (%P) of each fraction were then quantified.

2.4. Inhibition of ACE by PAs extracts and by Enalapril Maleate

The study of ACE inhibition by PAs was carried out testing purified fractions of seed and skin (1×10^{-3}, 5×10^{-3}, 1×10^{-2}, 0.1, 1 mg/ml) and Enalapril Maleate (0, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3} mg/ml) as inhibitors. The test was based on the hydrolysis of HHL by ACE to form HA (Eriz et al., 2011). ACE inhibition was quantified by determining the extract concentration and enalapril maleate (μM) necessary to produce an enzyme inhibition of the 50% of its maximum enzyme activity (IC_{50}). The IC_{50} was then related with the compositional and size characteristics of each extract in order to identify dependent relations and to determine if their inhibitory power was more influenced by the molecular size or by the molecular composition.

2.5. Statistical analysis

Purification and sample analyses were run in triplicate and the results were expressed as the mean value standard deviation. Statistical analyses were performed with Statgraphics plus 4.1 software package. To determine IC_{50} (μM), PA inhibitory capacity over ACE, the data were extrapolated using the Table Curve software (trial version).

3. Results and discussion

3.1. Chemical characterisation of PAs extracts

Once the *País* grapes were processed and the seeds/skin were separately extracted, the recovered seed mass (57 ± 0.15 mg d.w./grape) was found to be higher than skin mass (46 ± 0.05 mg d.w./grape). A comparison of the amount of seeds and skin of others varieties showed that *País* grapes have more skin than Merlot and Pinot Noir and less seed than Merlot (Cerpa-Calderon & Kennedy, 2008). After purification, the overall mass recovered from both sources was similar: 0.026 g skin extract/g skin versus 0.028 g seed extract/g seed; however, the mass proportion recovered among the fractions differed (Table 1). For both extracts, the first eluted fractions had less than 10% of mass, and the last ones

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