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An enzymatic extraction of proanthocyanidins from *País* grape seeds and skins

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ABSTRACT

Proanthocyanidins (PAs) from the skins and seeds of *País* grapes were obtained by enzymatic extraction by using three enzymes (pectinase, cellulase and tannase) and an enzymatic blend to increase the phenol concentrations and reduce the PA molecular size. The total phenol concentrations (as indicated by Folin–Ciocalteu reagent), mean degree of polymerisation (mDP), galloylation percentage (%G) and structural proportion (phloroglucinolysis) were analysed, in addition to the extract's capacity to inhibit angiotensin I-converting enzyme (ACE) activity. On grape skins, pectinase had the greatest effective on the release of total phenols, to 0.01 g/ml solid/liquid (S/L) and 1% enzyme/substrate (E/S). On grape seed, the three enzymes were effective in increasing the phenolic extraction ($p < 0.05$). The effects of enzymes on the mDP and %G of the extracts were related to their enzymatic activity. All the extracts inhibited ACE, but ACE inhibition was thought to be improved by the increased number of terminal units in the seed samples.

1. Introduction

Proanthocyanidins (PAs) in grape berries are located in both skins and seeds, and PA composition and concentration vary depending on the tissue of origin. Seeds contain a higher concentration of PAs than skin and a higher proportion of galloylated procyanidins, whereas skins contain prodelphinidins (Prieur, Rigaud, Cheynier, & Moutounet, 1994). In addition, skin PAs have been shown to have a higher mean degree of polymerisation (mDP) than proanthocyanidins from seeds (Eriz, Sanhueza, Roeckel, & Fernández, 2011).

In grape skins, PAs are synthesized in the cytoplasm and stored in the vacuole. They can also be associated with cell walls, where they are bound to polysaccharides (Busse-Valverde et al., 2010). In seeds, PAs are located in the seed coat (Geny, Saucier, Bracco, Daviaud, & Glories, 2003). Cell walls are a barrier to the diffusion of intracellular phenolic material for intact cells (Pinelo, Arnous, & Meyer, 2006). Studies on the extraction of skin PAs in model hydroalcoholic solution have shown that extraction is incomplete, with only 23% of the available skin PAs recovered in 12% v/v ethanol solution (Fournand et al., 2006). Comparisons of the extractable and inextractable PA composition and mean degree of polymerisation (mDP) indicated that extractable PA had a lower mDP, and inextractable PA had both higher mDP and higher subunit galloylation (Fournand et al., 2006). This work suggests that one limitation of PA extractability is its mDP and (potentially) its galloylation. Thus, extracting skin and seed PAs requires that the cell walls be broken to allow their vacuolar contents to be extracted or to diffuse into the medium. The degradation of cell-wall polysaccharides is a fundamental step to improve the release of phenols. Cellulases, hemicellulases, pectinases and other enzymes able to catalyse the hydrolysis of bonds in plant cell-wall polysaccharides can be employed to decompose the cell-wall structure (Pinelo et al., 2006).

The use of enzymes is now being expanded into various targeted applications beyond the classic press yield and clarification purposes in the wine (Busse-Valverde et al., 2010) and juice industries (Laaksonen et al., 2012). Arnous and Meyer (2010) studied the release of anthocyanins, flavonols (rutin and quercetin), phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) during the enzymatic (pectinolytic and cellulolytic) degradation of the cell wall polysaccharides in skins from Merlot and Cabernet Sauvignon wine grapes. However, they did not report the liberation of PAs. These authors concluded that enzymatic treatment with multicomponent pectinolytic enzymes may promote the discriminated release of phenols from grape skins and molecular changes in the phenols. Recently, Chamorro, Viveros, Alvarez, Vega, and Brenes (2012) reported changes in the polyphenol and polysaccharide contents of grape seed extract from enzymes. Nevertheless, the

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action of enzymes on PAs from the skins of grapes has not been reported.

PAs are among the most abundant polyphenols in our diet. The daily intake of flavan-3-ols in the United States has been estimated at approximately 60 mg/day for PAS with an mDP < 2 (Gu et al., 2004). In the Spanish population this it has been estimated to be 18–31 mg/day when considering proanthocyanidins with an mDP up to 3 (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000) and 450 mg/day for highly polymerised PAs (Saura-Calixto, Serrano, & Goni, 2007).

The bioavailability of proanthocyanidins is largely influenced by their degree of polymerisation and galloylation (de Pascual-Teresa, Moreno, & Garcia-Viguera, 2010; Monagas et al., 2010). Although monomeric flavan-3-ols are readily absorbed in the small intestine, oligomeric and polymeric forms pass intact through the gastrointestinal tract and reach the colon, where they must be transformed by the intestinal microbiota before absorption (de Pascual-Teresa et al., 2010). Thus, a reduction in the proanthocyanin mDP would be desirable, and one way to achieve this reduction could be the use of a specific enzyme that helps to increase the liberation of PA from seed and skin matrices while simultaneously reducing its average molecular weight.

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 characterised by its roughness and unbalance (FUCOA., 2012). 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.

One bioactivity reported for the PAs from *País* grapes is their inhibitory effect on angiotensin I-converting enzyme, ACE (Eriz et al., 2011; Fernandez & Labra, 2013; Godoy, Roeckel, & Fernandez, 2012). 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. 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 objective of this study was to collect and characterise the PAs obtained by enzymatically hydrolysing grape skins and seeds by using three enzymes (pectinase, cellulase and tannase) and their sequential blends, seeking an increase in the released phenol concentration and a decrease in the molecular size of the PAs. The skin and seed extracts were analysed to determine their total phenol concentrations (by Folin–Ciocalteu method), the mean degree of polymerisation (mDP) after acid catalysis in the presence of an excess of phloroglucinol (phloroglucinolysis), the galloylation percentage (%G), the structural molar proportion and the ACE inhibitor activity of the extracts.

2. Materials and methods

2.1. Materials

Glacial acetic acid, sodium carbonate, sodium acetate, methanol, hydrochloric acid and ethanol (Merck, Darmstadt, Germany) were used to perform the experiments. Water was purified in the laboratory by a Milli-Q plus, system (Millipore, Bedford, MA, USA). The acetic acid, acetonitrile and trifluoroacetic acid (TFA) used in the phloroglucinol catalysis were obtained from at 98% purity, HPLC grade (Merck, Darmstadt, Germany). 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, St. Louis, MO, USA. The three enzymes in the enzymatic extraction were obtained from Sigma–Aldrich (St. Louis, MO, USA); they included cellulase (code C1184); tannase (code 42395); and a mixture of pectinases sold under the trade name "Pectinex Ultra SPL" (code p2611), which is an active pectolytic enzyme preparation produced by a selected strain of *Aspergillus aculeatus* that primarily contains pectintranseliminase, polygalacturonase and pectinesterase, as well as small amounts of hemicellulases and cellulases. The main features of these enzymes are shown in Table 1.

Table 1
Characteristics of the enzymes[a] used in these experiments.

Enzyme name	Activity	pH optimun	T (°C) optimum	Source
Pectinex Ultra SPL	3800 ⩾ U/ml	4.0	25	*Aspergillus aculeatus*
Cellulase	0.3 ⩾ U/mg	5	37	*Aspergillus niger*
Tannase	150 ⩾ U/g	4.7	30	*Aspergillus ficuum*

[a] Information supplied by product sheets from Sigma–Aldrich.

2.2. Grape preparation

The *País* grapes were collected on March 2012 from the Biobío region of Chile. The grapes were separated from the stalk and immediately stored (−20 °C) in polyethylene bags. The grapes were thawed, and the grape skins and seeds were manually removed from the pulp. They were rinsed with distiled water at 4 °C and then lyophilized by using a Lyovac GT 2 freeze dryer (Leybold-Heraeus, Germany). The skins and dry seeds were ground separately in a mill (M20 Universal, Lurch, Germany) to obtain a particle size between 1 and 2 mm. Then, the samples were kept in the presence of silica gel in closed plastic bags wrapped with Kraft paper to prevent oxidation at -20 °C until use.

2.3. Enzymatic extraction of grape seeds and skins

The enzymatic extraction of lyophilized grape seed and skin samples was performed in a thermostatically controlled orbital shaker (New Brunswick Scientific G-24, USA) with gentle agitation (150 rpm) in a New Brunswick G24 gyratory shaker (New Brunswick Scientific Co., Edison, NJ, USA) for 3 h in the dark to prevent oxidation, in accordance with the reaction pH and temperature indicated in Table 1. The influence of the solid/liquid (S/L) dose on the grape skin extraction was studied for S/L 0.01 and 0.02 g/ml. In addition, the enzyme dose (E/S, 0%; 0.5%; 1%; 4%; 10%) was evaluated for pectinase, cellulase and tannase. A 0.5 g lyophilized grape skin or seed sample was extracted in 50 mL of buffer according to the enzyme under evaluation, and the enzyme preparation was added at the required enzyme/substrate ratio (%E/S) as calculated on a dry matter basis.

At the end of each experiment, 100 mL equivalent to 50:50 v/v of ethanol (100%) and acetate buffer with same pH to that of the enzyme was added to halt the enzymatic reaction. The sample was vortexed for 30 s, followed by centrifugation at 2512×*g* for 10 min at 4 °C. The supernatant was then filtered with a 0.45 μm nylon membrane and concentrated in a rotavap (Bibby Sterilin Ltd., RE-100B, Stone Staffordshire, England), to temperature (<35 °C) and pressure reduce to 15 mL. The concentrate was kept in a dark glass flask at −20 °C until the time of analyses. A control assay was carried out by following the same procedure as