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Role of cell wall deconstructing enzymes in the proanthocyanidin–cell wall adsorption–desorption phenomena

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ABSTRACT

The transference of proanthocyanidins from grapes to wine is quite low. This could be due, among other causes, to proanthocyanidins being bound to grape cell wall polysaccharides, which are present in high concentrations in the must. Therefore, the effective extraction of proanthocyanidins from grapes will depend on the ability to disrupt these associations, and, in this respect, enzymes that degrade these polysaccharides could play an important role. The main objective of this work was to test the behavior of proanthocyanidin–cell wall interactions when commercial maceration enzymes are present in the solution. The results showed that cell wall polysaccharides adsorbed a high amount of proanthocyanidins and only a limited quantity of proanthocyanidins could be desorbed from the cell walls after washing with a model solution. The presence of enzymes in the solution reduced the proanthocyanidin–cell wall interaction, probably through the elimination of pectins from the cell wall network.

1. Introduction

Proanthocyanidins (commonly called tannins by winemakers) are one of the main determinants of red wine quality. Some sensory attributes such as color, body and astringency are directly associated with the qualitative and quantitative composition of wine proanthocyanidins (PAs). In the grape, they are located in the skin and seeds and are transferred to the must/wine during the maceration step of winemaking. However, previous studies have shown that the quantities found in wines are frequently lower than expected (Adams & Scholz, 2007; Busse-Valverde, Bautista-Ortín, Gómez-Plaza, Fernández-Fernández, & Gil-Muñoz, 2012; Busse-Valverde et al., 2010), which may not only be related to the fact that PAs are not extensively extracted from the skin and seeds but also to the finding that a substantial proportion of them are adsorbed by the skin and pulp cell walls (CWs) in suspension in the must after crushing the grapes and that finally precipitates during settling (Bindon, Smith, Holt, & Kennedy, 2010).

The existence of interactions between proanthocyanidins and cell wall material, more precisely with the polysaccharides that are the main components of the cell walls, has been demonstrated and investigated by several research groups (Cai, Gaffney, Lilley, & Haslam, 1989; McManus et al., 1985; Riou, Vernhet, & Doco, 2002) as has the possible enological implications such interactions may have (Bautista-Ortin, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Bindon, Smith, Holt, et al., 2010; Le Bourvellec, Bouchet, & Renard, 2005). Therefore, the effective extraction of proanthocyanidins from grapes will depend on having enough knowledge of the nature of these interactions and on the ability to disrupt or manage these associations. As regards the nature of these associations it has been found that electrostatic or ionic interactions do not appear to play any part in the association between proanthocyanidins and cell-wall material, rather the adsorption mechanism seems to involve the establishment of weak interactions, more precisely hydrogen bonds and hydrophobic interactions (Le Bourvellec, Le Quere, & Renard, 2007). Studies have pointed to an increase in association with increasing ionic strength, suggesting the presence of hydrophobic interactions, and a notable decrease with increasing temperature, indicating hydrogen bonding (Le Bourvellec, Guyot, & Renard, 2004). Renard, Baron, Guyot, and Drilleau (2001) also demonstrated that washing the proanthocyanidins + CW complexes with 8 M urea, a chaotropic reagent, or an acetone:water 60:40 solution, resulted in total re-extraction of the procyanidins, while washing with buffer led only to partial re-extraction and adding NaCl actually decreased this

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re-extraction. All of this indicated that the bonds involved were most probably H-bonds or hydrophobic interactions, while ionic interactions were apparently absent.

During winemaking neither high temperatures nor urea can be used to limit the interactions. However, maceration enzymes, a very common enological product, may have a significant role on the extent of these interactions. Maceration enzymes may be used for improving must volume and clarification, for enhancing filterability and, especially in red winemaking processes, for increasing the degradation of the skin cell walls, the limiting barrier for the extraction of phenolic compounds. They exert their effect by deconstructing the polysaccharide network of the cell wall and allowing the extraction of the phenolic compounds located inside the vacuoles. However, the effect of these enzymes over the complexes that PAs and cell walls (CWs) form, whether they limit or favor the interactions and/or participate in the desorption processes of these compounds from cell walls have not been deeply studied.

2. Material and methods

2.1. Chemicals

Chromatographic solvents were of high-performance liquid chromatography (HPLC) grade, and chemicals were of analytical reagent grade. Acetonitrile, acetone, chloroform, methanol, ethanol, formic acid and trifluoroacetic acid were from Merck (Darmstadt, Germany). The phloroglucinol reagent and tris–HCl equilibrated phenol pH 6.7 were sourced from Sigma Aldrich (MO, USA). Sodium acetate was from J.T. Baker (Deventer, Netherlands). The standards (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate and (−)-epigallocatechin were obtained from Extrasynthese (Genay, France). Galacturonic acid was from Sigma (St. Louis, MO, USA) and Bovine serum albumin (BSA) fraction V from J.T. Baker (Deventer, Holland). For the glucose determination, a enzymatic analysis kit from R-biopharm (Darmstadt, Germany) was used.

2.2. Instrumentation

The HPLC apparatus was a Waters 2695 (Waters, Milford, MA) equipped with a Syrahstem autosampler, and a Waters 2996 photodiode array detector (Waters, Milford, MA).

2.3. Grapes

Vitis vinifera L. cv. Monastrell grapes were sampled from a commercial vineyard located in Jumilla, Murcia (Spain). Grapevines grafted in 2005 onto 110R rootstock trained to a bilateral cordon system and trellised to a three-wire vertical system. The vineyard was drip irrigated. Grapes were sampled at two stages of ripeness, veraison (14 °Brix) and harvest (25 °Brix), for the 2013 growing season. To obtain a representative vineyard sample, a 1000-berry sample was collected from three rows distributed within the vineyard block, and then pooled and transported to the laboratory, where they were stored at −20 °C until analysis.

2.4. Cell wall material

Purified cell walls (CW) were extracted from fresh skins of *V. vinifera* L. cv. Monastrell. Cell walls were isolated following the method of De Vries, Voragen, Rombouts, and Pilnik (1981) and adapted by Ruiz-García, Smith, and Bindon (2014). Briefly, skins were extracted in 70% v/v acetone to remove proanthocyanidins. The acetone-extracted residues were washed in additional 70% v/v acetone, followed by Milli-Q water. Acetone-extracted skin material was then homogenized under liquid nitrogen. Thereafter, acetone-insoluble residues from skins (1 g) were extracted in 50 mL of tris–HCl equilibrated phenol pH 6.7, and then washed in 80% v/v methanol (100 mL), and in acetone (100 mL) to remove phenol. Samples were then extracted with slow shaking for 30 min in 1:1 v:v methanol/chloroform (50 mL) and washed in methanol (100 mL), and in acetone (100 mL). The insoluble residue was then lyophilized. Recovered CW was manually ground to a fine particle size with a mortar and pestle and then frozen at −20 °C until used.

2.5. Analysis of cell wall composition

Uronic acids were determined in the sulfuric acid cell wall hydrosilate by the colorimetric 3,5-dimethylphenol assay after cell walls pretreatment (30 °C, 1 h) with aq. 72% sulfuric acid followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Pure galacturonic acid was used as a standard.

The proteins and total phenolic compound content of the cell wall material were determined after extraction with 1 M NaOH (100 °C, 10 min) by the colorimetric Coomassie Brilliant Blue assay and by the colorimetric Folin–Ciocalteau reagent assay, respectively. Bovine serum albumin (BSA) fraction V and pure gallic acid were used as standards, respectively.

The total glucose was determined using a kit for glucose enzymatic analysis from R-biopharm (Darmstadt, Germany) after pretreatment (30 °C, 1 h) with aqueous 72% sulfuric acid, followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Hydrolysis using only 1 M sulfuric acid (100 °C, 3 h) was used to determine non-cellulosic glucose. Cellulosic glucose was obtained by difference between the total glucose and non-cellulosic glucose content. The acid-insoluble residue obtained after pretreatment and hydrolysis was used to estimate the content of lignin (Klason lignin).

2.6. Proanthocyanidins used in the interaction studies

The proanthocyanidin used for the experience was seed-derived commercial tannin (TanReactive, Agrovin, Alcazar de San Juan, Spain) with a purity of 56%, an degree of polymerization of 2.77 and a galloylation percentage of 7.5%. The purity of the commercial tannin was estimated spectrophotometrically following the method of Ribéreau-Gayon, Glories, Maujean, and Dubourdieu (1998) after acid hydrolysis of the samples and the degree of polymerization and galloylation percentage were calculated with the phloroglucinolysis method described in Section 2.8.

2.7. Binding reactions between tannins and cell wall material with and without enzyme

Test 1: Skin CWs from veraison and ripen grapes were separately weighed into 3 mL tubes. CW samples were then combined with the enological tannin previously dissolved in a model solution (12% ethanol and pH 3.6 adjusted with trifluoroacetic acid) at a concentration of 2 g/L. The reaction volume was 2.5 mL and the final CW concentration was 13 mg/mL. The samples were shaken at 300 rpm in an orbital shaker at room temperature for 90 min. Six replicates were performed for each state of ripeness of the cell walls. A blank without CW was also included. After the binding reaction, samples were centrifuged at 13,000 rpm and the supernatant was transferred to a new tube. Samples were then dried under vacuum at 35 °C. Recovered tannin was reconstituted in 250 μL of methanol and then analyzed by phloroglucinolysis and size exclusion chromatography (SEC). The precipitates comprising the CW–proanthocyanidin complexes were redissolved, in triplicate, with 2.5 mL of the model solution containing a commercial enzyme (Enozym Vintage, supplied by Agrovim S.A., Spain, at a