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Analysis of the Oxidative Degradation of Proanthocyanidins under Basic Conditions

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Proanthocyanidin isolates from grape (*Vitis vinifera* L. cv. Pinot noir) skin and seed underwent oxidative degradation in solution (10 g/L) under basic conditions while exposed to atmospheric oxygen. Degradation was monitored by reversed-phase HPLC following acid-catalyzed cleavage in the presence of excess phloroglucinol (phloroglucinolysis) and by high-performance gel permeation chromatography. All isolates degraded under these conditions and followed second-order kinetics for over 1 half-life, consistent with an oxidation reaction. The conversion of proanthocyanidins to known subunits (conversion yield) when measured by phloroglucinolysis showed a dramatic decline over the course of the reaction. With the exception of (+)-catechin extension subunits, all individual subunits decreased in concentration during the oxidation process, also following second-order kinetics for over 1 half-life. Skin proanthocyanidins degraded the fastest due to the presence of (−)-epigallocatechin extension subunits. Seed procyanidins were degraded with and without flavan-3-ol monomers. Flavan-3-ol monomers slowed the rate of seed procyanidin degradation. The mean degree of polymerization (mDP) determined by phloroglucinolysis indicated a large decrease in mDP as the reaction progressed; yet, by GPC, the size distribution of all proanthocyanidins changed little in comparison. The conversion yield could be an important parameter to follow when using phloroglucinolysis as a means for determining proanthocyanidin mDP, and when monitoring the oxidative degradation of proanthocyanidins.

KEYWORDS: Proanthocyanidins; condensed tannins; oxidation; composition; size distribution; *Vitis vinifera*; grape; seed; skin

INTRODUCTION

Proanthocyanidins are natural polymeric plant products composed of flavan-3-ol subunits. Proanthocyanidin structure varies considerably, with subunit, interflavonoid bond location and branching, as well as modification with nonflavonoid substitutes and molecular weight distribution contributing to this variation (*1*). Proanthocyanidins are important in many foods and beverages due to their bitter and astringent properties, and there is increasing interest in this class of compounds because of their potential role in human health (*2*).

Oxidation is an essential process in the production of many phenolic-containing foodstuffs, including red wine, where it has long been considered that limited oxygen exposure throughout the winemaking process leads to improved wine quality (*3*). Because of their bitter and astringent properties, proanthocyanidins are important components in wine (*4*). Their concentration combined with their phenolic substitution pattern makes them major substrates for oxygen in wine (*5*−*7*).

The effective management of oxidation during wine production requires an understanding of how wine chemistry changes during oxidation. Oxidation is expected to affect proanthocyanidin structure, and in turn these changes are likely to lead to modifications in proanthocyanidin sensory properties. To better understand this relationship, there is a need to develop our understanding of proanthocyanidin reactivity and structural change when oxidation occurs.

The purpose of this study was to understand how proanthocyanidins isolated from grape seeds and skins changed under controlled oxidative conditions. Given that the rate of phenolic oxidation increases with pH (*5*, *7*), these studies were conducted under mildly basic conditions to reduce the study time. Two recently described chromatographic methods were used to analyze the proanthocyanidins during oxidation because it was thought that they could provide additional information on proanthocyanidin structure change during oxidation (*8*, *9*).

Although the ultimate goal of this research is to understand how proanthocyanidins in wine change during oxidation, wine is a complex medium. Therefore, a simplified model system composed of *N*,*N*-dimethylformamide and methanol was used to understand the chemical reactions, without the introduction

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Table 1. Individual Subunit Concentration, Conversion Yield, and Mean Degree of Polymerization for Proanthocyanidin Isolates

	extension subunits				terminal subunits[a]				
sample	EGC[b]	C	EC	ECG	C	EC	ECG	yield[c]	mDP[d]
skin	7.36×10^{-3}	8.86×10^{-4}	1.67×10^{-2}	2.04×10^{-4}	1.18×10^{-3}			0.77	22.3
seed		2.78×10^{-3}	1.69×10^{-2}	7.62×10^{-4}	1.47×10^{-3}	5.93×10^{-4}	7.86×10^{-4}	0.67	8.1
seed + monomer		2.79×10^{-3}	1.59×10^{-2}	8.11×10^{-4}	3.73×10^{-3}	1.80×10^{-3}	7.12×10^{-4}	0.75	4.1

[a] Includes flavan-3-ol monomers. [b] Molar concentration of individual subunits in reaction solution, and with the following subunit abbreviations: EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate. [c] Conversion yield (w/w) in the conversion of proanthocyanidin isolate into known subunits. [d] Mean degree of polymerization.

of potential physical instability issues. The results of this study can then be compared in future research using a wine medium.

MATERIALS AND METHODS

Chemicals. Acetone, hexane, ethyl acetate, acetonitrile, dichloromethane, *N*,*N*-dimethylformamide, and methanol were HPLC grade and purchased from Fisher Scientific (Santa Clara, CA). Also purchased from Fisher Scientific were glacial acetic acid, hydrochloric acid, potassium hydroxide, L-(+)-ascorbic acid, lithium chloride, and sodium acetate, all of which were reagent grade. Phloroglucinol (reagent grade) was purchased from Sigma (St. Louis, MO). Trifluoroacetic acid (reagent grade) was obtained from Aldrich (St. Louis, MO). Toyopearl HW-40F chromatography media was obtained from Supelco (St. Louis, MO). The distilled or reverse-osmosis water used in all solutions was purified to HPLC grade with use of a Millipore Milli-Q water system.

Proanthocyanidin Isolation and Purification. *Vitis vinifera* L. cv. Pinot noir grape berries grown during the 2002 harvest season at Oregon State University experimental vineyard were used as the source material for proanthocyanidins. Samples were randomly selected from one vineyard block approximately three weeks prior to véraison. The skins of the grapes were manually separated from the seeds and kept at 4 °C until extracted.

For extraction, skins and seeds were placed in covered Erlenmeyer flasks with 2:1 v/v acetone:water (~1:1 v:v liquid:fruit) at room temperature for 24 h. Afterward, the extract was filtered through no. 1 Whatman filter paper to remove seed and skin tissue, and the acetone was then removed under reduced pressure at 38 °C. The aqueous extract was then extracted three times with hexane (3:1 v/v extract:hexane) to remove lipophillic material, followed by extracting three times with ethyl acetate (4:1 v/v sample:ethyl acetate) to remove low molecular weight phenolic compounds. The extract was concentrated under reduced pressure at 38 °C, and then lyophilized to a dry powder. This extraction procedure is based upon a previously described procedure (*8*, *9*).

Crude proanthocyanidins were purified by adsorption chromatography, using Toyopearl TSK 40-F size exclusion media. The column was equilibrated with 1:1 v/v methanol:water containing 0.05% v/v trifluoroacetic acid. The crude proanthocyanidin isolate was dissolved in a minimal amount of this mobile phase and applied to the column. The column was rinsed with 5 volumes of the mobile phase to remove carbohydrate and low molecular weight phenolics. To prepare a proanthocyanidin isolate containing flavan-3-ol monomers (seed + monomer), the column was rinsed with 3 volumes of mobile phase instead of 5 volumes (seed). Proanthocyanidins were then eluted with 3 volumes of 2:1 v/v acetone:water containing 0.05% v/v trifluoroacetic acid. The eluent was concentrated under reduced pressure at 38 °C to remove acetone and then lyophilized to a dry powder.

Proanthocyanidin Oxidation. Purified proanthocyanidins (20 g/L) were prepared in solutions consisting of 20% v/v methanol and 80% v/v *N*,*N*-dimethylformamide (DMF). A potassium hydroxide solution (0.0323 M) was prepared in 20% methanol and 80% DMF. To start the reaction, equal volumes of the proanthocyanidin- and KOH-containing solutions were combined into 20-mL flint glass vials equipped with rubber lined caps, leaving about 10 mL of air space. Reaction solutions were mixed well, and samples were taken regularly over 33 h (samples taken after cap removal), quenched, and then analyzed to determine proanthocyanidin composition and size distribution.

Acid Catalysis in the Presence of Phloroglucinol Followed by Reversed-Phase HPLC (Phloroglucinolysis). Samples were analyzed by reversed-phase HPLC following acid-catalyzed cleavage in the presence of excess phloroglucinol (phloroglucinolysis), using a previously described method (*8*, *9*), although several changes were made. These changes included the following: the cleavage reagent was concentrated to allow for dilution when combined with the proanthocyanidin-containing solution (1 volume of proanthocyanidin solution was combined with 1 volume of reagent). Additional acid (HCl) was also added to neutralize the KOH in the reaction mixture. The reagent therefore consisted of 0.216 N HCl in MeOH, containing 100 g/L of phloroglucinol and 20 g/L of ascorbic acid. The reaction was carried out at 50 °C for 20 min, and then combined with 5 volumes of 40 mM aqueous sodium acetate to stop the reaction. An Agilent 1100 HPLC instrument (Palo Alto, CA, USA), which consisted of a vacuum degasser, pump, autosampler, column oven, and diode array detector, was used for all analyses. This method provided information on subunit composition, conversion yield, and mean degree of polymerization, all of which were determined as previously described (*9*).

Gel Permeation Chromatography. The size distribution of proanthocyanidins was determined by high-performance gel permeation chromatography (GPC), using a previously described method (*8*). Prior to analysis, the proanthocyanidin-containing solution was combined with DMF containing 2 equiv of acetic acid to neutralize the KOH in the reaction mixture (1 volume of proanthocyanidin solution was combined with 1 volume of acetic acid containing DMF).

RESULTS AND DISCUSSION

The purpose of this investigation was to monitor proanthocyanidin size and composition under reaction conditions that would favor oxidation (i.e., basic conditions). In this study, three isolates were studied: an isolate from skin tissue (skin), an isolate from seed tissue (seed), and an isolate from seed tissue containing additional flavan-3-ol monomers (seed + monomer).

Initial Proanthocyanidin Composition. Proanthocyanidin isolates were characterized by phloroglucinolysis to determine the subunit composition, conversion yield, and mean degree of polymerization (mDP **Table 1**). All isolates were composed primarily of (−)-epicatechin extension subunits. Skin also contained a significant proportion of (−)-epigallocatechin extension subunits and a low amount of monomeric material. The compositional data were consistent with previously published data (*10*−*12*).

After isolation and purification of the seed and skin proanthocyanidins, it was confirmed that skin and seed isolates contained little if any residual flavan-3-ol monomers, while seed + monomer contained a significant amount including (+)-catechin and (−)-epicatechin (**Table 1**). Due to a combination of ethyl acetate extraction and partial elution of flavan-3-ol monomers during column purification, the proportion of monomeric material in seed + monomer was lower than that in previous studies (*11*).

The conversion yields ranged from 67% to 77% (w/w) with skin having the highest conversion yield. These yields are consistent with previous results with this method (*9*). The mean

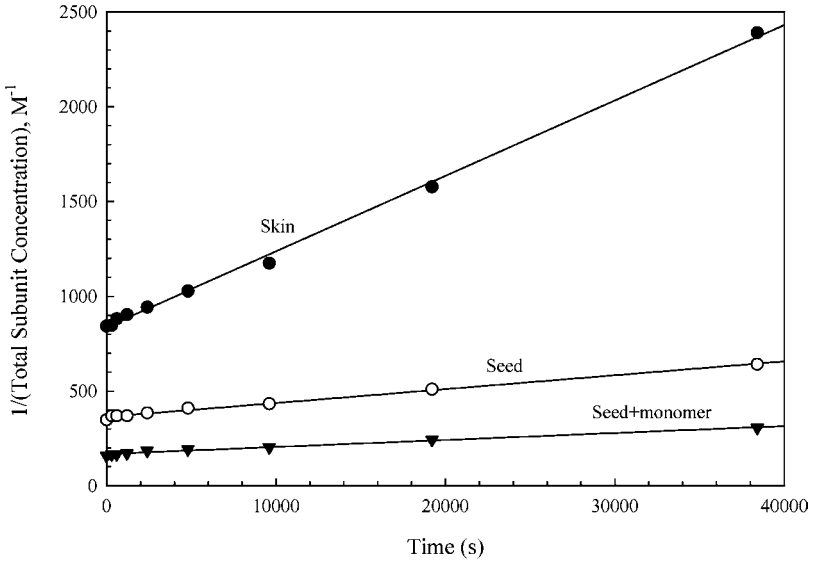

Figure 1. Second-order plot of the total subunit concentration derived from proanthocyanidin isolates upon degradation under basic conditions.

Table 2. Individual Subunit Rate Constants[a] for the Three Proanthocyanidin Isolates

	extension subunits				terminal subunits[b]		
sample	EGC[c]	C[c]	EC[c]	ECG[c]	C[c]	EC[c]	ECG[c]
skin	2.05×10^{-2} [d] (0.98)	*e*	1.99×10^{-3} (0.98)	1.89×10^{-1} (0.95)	1.76×10^{-2} (0.98)	*f*	*f*
seed	*f*	*e*	1.59×10^{-3} (0.99)	1.06×10^{-1} (0.99)	1.03×10^{-2} (0.97)	1.69×10^{-2} (0.94)	2.63×10^{-2} (0.95)
seed + monomer	*f*	*e*	2.32×10^{-3} (0.99)	1.48×10^{-2} (0.99)	1.81×10^{-3} (0.90)	4.19×10^{-3} (0.90)	2.70×10^{-2} (0.99)

[a] Correlation coefficient indicated in parentheses. [b] Includes flavan-3-ol monomers. [c] Subunit abbreviations: EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate. [d] Observed second-order rate constant (M^{-1} s^{-1}). [e] Did not follow second-order order kinetics. [f] Not observed.

degree of polymerization of the isolates differed considerably. Given that the reaction solutions were prepared on an equal mass basis, and because the mDP for each isolate was different, the estimated molarity of the individual reaction solutions was different. This was calculated by dividing the total subunit concentration (M) by the mDP, to arrive at an estimated proanthocyanidin concentration (M). The estimated molar concentration was highest for seed + monomer (6.28×10^{-3} M), followed by seed (2.87×10^{-3} M) and skin (1.18×10^{-3} M).

Proanthocyanidin Reaction. All isolates degraded under the reaction conditions (**Figure 1**) resulting in a considerable visual change during the monitoring time. Initially proanthocyanidin solutions were light orange in color increasing in darkness to brown-black by the end of the reaction (33 h). No visual precipitate was observed in any of the reactions, confirming that the solvent system selected was effective in maintaining physical stability. After 21 h, the conversion yields had declined considerably to a low of 11.8% for skin and 25.1% and 28.2% for seed and seed + monomer, respectively. These results are consistent with oxidation. The dramatic decline in conversion yield over the reaction time suggests that this parameter could be an important indicator for overall reaction progress.

Reaction progress was also determined by measuring subunit loss (total and individually). All reactions followed second-order kinetics for at least 1 half-life, consistent with previous studies (*13*−*15*). The data deviated from second-order kinetics after 1 half-life. Half-lives for proanthocyanidin isolates (total subunit concentration for the first half-life) were calculated ($t_{1/2} = 1/(k[A]_0)$). Skin degraded fastest (3.98×10^{-2} M^{-1} s^{-1}, $R^2 = 0.998$) followed by seed (7.31×10^{-3} M^{-1} s^{-1}, $R^2 = 0.994$) and seed + monomer (3.68×10^{-3} M^{-1} s^{-1}, $R^2 = 0.986$). Skin had the shortest half-life (5.9 h) followed by seed + monomer (12.0 h) and seed (13.2 h).

Individual Subunit Reaction. To explore differences within each of the proanthocyanidin isolates, the individual subunit degradations within each of the isolates were compared. All subunits followed second-order kinetics beyond 1 half-life with the exception of (+)-catechin extension subunits, which apparently increased during the course of the reaction (**Table 2**).

The deviation from second-order kinetics for (+)-catechin extension subunits may be explained by epimerization of (−)-epicatechin extension subunits to (−)-catechin and (+)-catechin to (+)-epicatechin, both of which are base-catalyzed reactions (*16*, *17*). The chromatography method used for analysis does not have the ability to resolve (+)-catechin and (−)-epicatechin from their C-2 epimers and, therefore, given that (−)-epicatechin comprised a greater proportion of the proanthocyanidin isolate and the rate of epimerization for (−)-epicatechin exceeds that of (+)-catechin (*16*), could explain the apparent increase in (+)-catechin during the reaction.

The rates of degradation of individual subunits varied for all isolates. The (−)-epicatechin-3-*O*-gallate extension subunits had the highest rate of degradation (**Table 2**); however, the overall concentration of this subunit was minor in all of the isolates (**Table 1**). For the skin, (−)-epicatechin and (−)-epigallocatechin extension subunits made up the majority of proanthocyanidin composition and (−)-epigallocatechin degraded more rapidly than (−)-epicatechin. Due to the presence of (−)-epigallocatechin extension subunits and the increased rate of degradation of skin (−)-epicatechin extension subunits (compared to (−)-epicatechin extension subunits contained in seed isolates), skin had the largest overall rate of degradation (**Figure 1**).

For the seed, the addition of monomeric material affected the rate of degradation of the individual subunits. For (+)-catechin and (−)-epicatechin terminal subunits (which included flavan-3-ol monomers), the rate of loss was reduced by 5.7 and

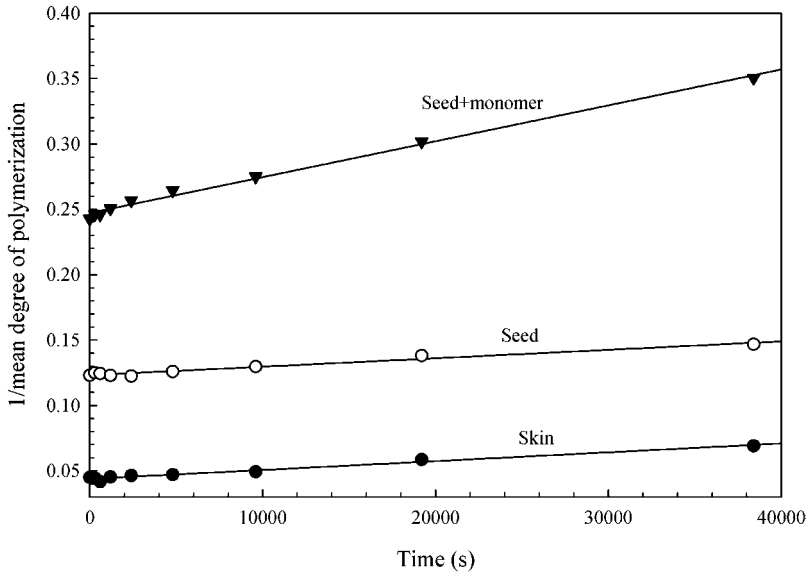

Figure 2. Second-order plot of proanthocyanidin mean degree of polymerization upon degradation under basic conditions.

4.0 times, respectively. One possible explanation for this observation is that the flavan-3-ol monomers reacted more slowly than (+)-catechin and (−)-epicatechin procyanidin terminal subunits. Additional studies with flavan-3-ol monomers quantified separately from proanthocyanidin terminal subunits would help to more fully understand this observation. As there was not an increase in the molar concentration of (−)-epicatechin-3-*O*-gallate terminal subunits in the seed + monomer system, the rate of degradation for (−)-epicatechin-3-*O*-gallate was similar.

The intramolecular environment of the proanthocyanidins in skin and seed may partially explain the observed difference in extension subunit reaction rates. An increase in the rate of loss for (−)-epicatechin extension subunits was observed when going from seed to skin. Given that the molar concentration of (−)-epicatechin extension subunits for skin was similar to that for seed suggests that the intramolecular environment affected the rate of reaction. The difference may be due to (−)-epigallocatechin extension subunits, in that they influenced the rate of degradation of (−)-epicatechin extension subunits (i.e., the apparent oxidation of (−)-epigallocatechin extension subunits may have affected the stability of adjacent (−)-epicatechin extension subunits).

While the intramolecular environment of the subunits within the proanthocyanidins of the seed and seed + monomer systems was the same, intermolecular interactions were different, due to differing overall proanthocyanidin (and flavan-3-ol monomer) molar concentrations. One potential explanation for the increase in extension subunit degradation when going from the seed to the seed + monomer system may be the increase likelihood of intermolecular interactions in the seed + monomer system (i.e., the seed + monomer system had a higher molar concentration that the seed system).

Proanthocyanidin Oxidation Half-Lives. The half-lives of the individual subunits were calculated by using the information from **Tables 1** and **2**. The longest half-life is over 20 times greater than the shortest half-life (**Table 3**). Overall, the half-life for the terminal subunits is longer than that for the extension subunits, despite their greater rates of degradation. This is due to differing initial molar concentrations (**Table 1**).

Proanthocyanidin mDP. Because of the longer half-lives (**Table 3**) for the terminal subunits and given the calculation for determining mDP (total subunit concentration/terminal subunit concentration; *9*), a large decrease in the mean degree of polymerization was observed in all isolates upon degradation. The rate of mDP change appeared to follow second-order kinetics (**Figure 2**). If proanthocyanidins are being oxidized, it is reasonable to conclude that additional, oxidatively generated interflavonoid bonds should be produced (*13*−*15*). Given this, it is also reasonable to conclude that proanthocyanidin mDP should increase when oxidized. This was not observed.

Table 3. Half-Life (min) of Individual Subunits for the Three Proanthocyanidin Isolates

	extension subunits				terminal subunits[a]		
sample	EGC[b]	C[b]	EC[b]	ECG[b]	C[b]	EC[b]	ECG[b]
skin	110	*c*	499	432	803	*d*	*d*
seed	*d*	*c*	620	206	1101	1663	806
seed + monomer	*d*	*c*	452	1389	2469	2210	867

[a] Includes flavan-3-ol monomers. [b] Subunit abbreviations: EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-*O*-gallate. [c] Not determined. [d] Not observed.

Table 4. Change in Proanthocyanidin Mean Degree of Polymerization and Average Molecular Weight Following Degradation

sample	% of initial mDP[a]	% of initial av mol wt[b]
skin	57.9	104.4
seed	71.8	93.2
seed + monomer	50.2	90.3

[a] mDP as determined by phloroglucinolysis. [b] Molecular weight equivalent to 50% elution when analyzed by GPC.

To provide additional information on how oxidation affected the size of proanthocyanidins an additional analytical method that measures size distribution (GPC) was utilized (*8*). The results were different than those determined by phloroglucinolysis (**Table 4**). For skin, and over the course of the reaction, there was a small increase in the predicted molecular weight at 50% mass elution (104.4% of $t = 0$) as determined by GPC but there was a dramatic decrease (57.9% of $t = 0$) as predicted by phloroglucinolysis. For seed and seed + monomer, although results from both analytical methods are consistent in terms of direction (i.e., both methods indicated a reduction in molecular size upon degradation), reductions in mDP as determined by

phloroglucinolysis were much greater than those measured by GPC. Overall, the changes in molecular size distribution as observed by GPC were much less than the changes in mDP observed by phloroglucinolysis.

It is clear that using phloroglucinolysis to determine accurate proanthocyanidin mDP relies on prior knowledge of structure (i.e., conversion yield, subunit composition, and interflavonoid bond information). Because we are currently unable to completely define proanthocyanidin structure, reported values for mDP by phloroglucinolysis should be accompanied by conversion yield information as well as assumed subunit composition and interflavonoid linkages. The small change in molecular size distribution as measured by GPC (despite the very large degree of oxidative degradation) suggests that proanthocyanidin size does not vary markedly when oxidized.

The overall results of this study provide new insights into the reactivity of proanthocyanidins under oxidative conditions and new approaches for monitoring these changes.

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