Enzyme release of phenolics from muscadine grape (Vitis rotundifolia Michx.) skins and seeds

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Abstract
Enzyme degradation of plant cell wall polysaccharides can potentially enhance the release of bioactive phenolics. The aim of this study was to evaluate various combinations of solvent and enzyme, enzyme type (cellulase, pectinase, ß-glucosidase), and hydrolysis time (1, 4, 8, 24 h) on the release of muscadine grape skin and seed phenolics, and their antioxidant activities. Results showed that pre-treated muscadine skins and seeds with enzymes decreased total phenolic yield compared with solvent (50% ethanol) alone. Enzyme release of phenolics from skins of different muscadine varieties was significantly different while release from seeds was similar. Enzyme hydrolysis was found to shorten extraction time. Most importantly, enzyme hydrolysis modified the galloylated form of polyphenols to low molecular weight phenolics, releasing phenolic acids (especially gallic acid), and enhancing antioxidant activity.

1. Introduction

Muscadine grape (Vitis rotundifolia Michx.) is an important grapevine species native to southeastern United States and Mexico, and has been extensively cultivated since the 16th century. Muscadine grapes are rich sources of bioactive phenolics and other nutrients studied for their potential health benefits. The compounds identified in muscadine seeds included hydroxybenzoic acid, hydrolyzable tannins, flavan-3-ols and condensed tannins, ellagic acid derivatives, and quercetin rhamnoside; the skin contained hydroxybenzoic acid, hydrolyzable tannins, flavonoids, including anthocyanin 3,5-diglucosides, ellagic acid derivatives, quercetin, myricetin, and kaempferol glycosides (Sandhu & Gu, 2010). Cell culture studies have suggested that phenolics from muscadine grapes have strong anticancer activities, such as inhibiting proliferation of colon and prostate cancer cells by inducing apoptosis (Hudson et al., 2007; Mertens-Talcott, Lee, Percival, & Talcott, 2006). Therefore, muscadine grape pomace is a potential source of bioactive phenolics, which could be used in the food and pharmaceutical industries.

In grape, phenolics in general can be classified as (1) cell-wall phenolics, which are bound to polysaccharides by hydrophobic interactions, hydrogen bonds and covalent bonds, and (2) non-cell-wall phenolics, encompassing phenolics confined in the vacuoles of plant cells and phenolics associated with the cell nucleus (Liyama, Lam, & Stone, 1994; Pinelo, Arnous, & Meyer, 2006). The cell wall of grape fruit is a complex network composed of about 30% neutral polysaccharides (cellulose, xyloglucan, arabinoxylan, galactan, xylan and mannan), 20% acidic pectin substances (of which 62% are methyl esterified), 15% insoluble proanthocyanidins, and <5% structural proteins (Lecas & Brillouet, 1994). Degradation of cell wall polysaccharides, which eliminates this physical barrier and opens up the cell, is a fundamental step in improving the release of phenolics from grape fruit. Research has focused on the application of cell wall hydrolyzing enzymes, such as cellulases, glucanase, and pectinases, to release phenolics from grape fruit and pomace. Kammerer, Claus, Schieber, and Carle (2005) reported that pectinases and cellulases could result in notably higher recovery rates of phenolics from Vitis vinifera L. grape pomace. Pectinases and macerating enzymes also were reported to promote anthocyanin extraction and improve the quality of red wines (Haight & Gump, 1994). However, others have found that pectinases and macerating enzymes can cause a decrease in the total yield of anthocyanins and a loss of wine color, or the pectinases have no apparent benefit (Wightman & Wrolstad, 1996; Wrolstad, Wightman, & Durst, 1994). Cellulase treatment was reported not effective for phenolic release from grape pomace.
(Vitis vinifera L.) pomace (Chamorroa, Viverosbl, Alvarezoa, Vegaan, & Brenesa, 2012). Li, Smith, and Hossain (2006) reported that enzyme-assisted aqueous extraction did not give as high a recovery of citrus peel phenolics as solvent (72% ethanol) extraction. With so many contradictory findings, and considering the phenolic profiles from different grape species/varieties are not the same (Li, Pan, Jin, Mu, & Duan, 2011; Li et al., 2009; Xu, Zhang, Cao, & Lu, 2010), an in-depth study of phenolic release by enzymes in various grape species would be useful.

Not only may hydrolysis of cell wall polysaccharides help release various phenolics, these enzymes may hydrolyze the polyphenols into low molecular weight phenolics, which may increase the availability and bioactivity of these phenolics (Chamorroa et al., 2012; Zheng, Hwang, & Chung, 2009). Monomeric and some oligomeric polyphenols have been found to absorb into rat plasma and are directly involved in physiological functions (Shoji et al., 2006), while polymeric forms are poorly absorbed (Donovan, Manach, Rios, Morand, Scalbert & Remesy, 2002). Therefore, it is important to evaluate the effect of enzyme hydrolysis of grape pomace on the magnitude of phenolic release, structure of phenolics after hydrolysis and antioxidant activities.

Additionally, muscadine skins are much thicker compared to any other varietal grapes. The thick skin accounts for about 40% of the fresh weight of the grape, which gives muscadine grapes a natural resistance to disease, fungi, and insects, and are where much of the antioxidant power of the muscadine grape is stored (Clayton, 1985). Enzyme degradation of muscadine skin cell wall polysaccharides has the potential to enhance the release of these bioactive phenolics.

To our knowledge, there has been little information on enzyme hydrolysis for the release of muscadine grape (Vitis rotundifolia Michx.) skin and seed phenolics (Lee & Talcott, 2005). This study comprehensively studied the enzymatic release of muscadine grape skin and seed phenolics by evaluating various combinations of solvent and enzyme, enzyme type (cellulase, pectinase, ß-glucosidase), and hydrolysis time (1, 4, 8, 24 h) on the release of muscadine grape skin and seed phenolics and their antioxidant activities.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Grape materials

Fully ripe muscadine grape (Vitis rotundifolia Michx.) cv. Noble (red) and cv. Carlos (bronze) were harvested from the Center for Viticulture and Small Fruit Research (latitude 30.65 N, longitude 84.60 W) at Florida A&M University on September 2, 2011. The collected samples were shipped to the University of Florida on the same day and stored in a cold room (4°C). Grape skins and seeds were separated manually from berries and freeze-dried in a freeze drier (Advantage, The Virtis Company, NY, USA) within the following three days. The freeze-dried samples were stored in vacuum-packaged polyethylene pouches at –20°C until analysis.

#### 2.1.2. Sample preparation

Freeze-dried grape skins (20 g) were ground with a stainless-steel grinder (Omni-Mixer 17105, OCI Instruments, CT, USA) for 1 min, and then placed on a sieve (≤0.25 mm) and the fine powder passing through the sieve collected (Xu, Zhang, Wang, & Lu, 2010). The powdered samples were stored at –20°C and used for subsequent analysis.

Freeze-dried grape seeds (20 g) were crushed and then defatted with hexane at a ratio of 1:10 (w/v). After 24 h of extraction at room temperature (shaking every each 6 h), the hexane extract was filtered using Whatman #4 filter paper (0.45 µm) (Fisher Scientific, Pittsburgh, PA) under vacuum. The residue was evenly distributed over a tray and kept in the hood to evaporate the hexane. The final defatted grape seed powder was ground again in the stainless-steel grinder and the powder through the sieve (≤0.25 mm) was collected. The samples were also stored at –20°C and used for subsequent analysis.

### 2.1.3. Chemicals

#### 2.1.3.1. Different combinations of solvent or enzyme.

A total of 10 combinations of solvent or enzyme were used for the phenolic extraction experiments (Table 1). Freeze-dried and ground skins or seeds (0.25 g) were weighed into a tube and extracted following the procedures in Table 1 with 2 or 5 mL of the listed solvents for 1 h at 50°C or room temperature. To investigate the yield at each step, three extractions were performed per sample. To examine the enzyme release of phenolics from grape skin or seed, 0.25 g of sample was incubated with a mixture of cellulase (100 U/g dry matter, 100 enzyme-activity units/g dry weight sample), pectinase (7 U/g dry matter) and β-glucosidase (25 U/g dry matter) in 2 mL of 0.2 M Na acetate buffer (pH 4.8) for 1 h at 50°C in a Microprocessor Controlled 280 Series water bath (Precision, Winchester, VA, USA). The selected hydrolysis conditions (pH 4.8 and 50°C) were based on evaluations of temperature and pH activity curves for the enzymes. This information was provided in the enzyme suppliers’ data sheets. The optimal enzyme concentration for each enzyme was selected in preliminary experiments by testing different con-

### 2.2. Methods

#### 2.2.1. Extraction of phenolic compounds

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<table>
<thead>
<tr>
<th>Condition</th>
<th>Extraction 1</th>
<th>Extraction 2</th>
<th>Extraction 3</th>
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<tr>
<td>#1</td>
<td>Water (2 mL)</td>
<td>Water (5 mL)</td>
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<td>#2</td>
<td>50% ethanol (2 mL)</td>
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<tr>
<td>#3</td>
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<tr>
<td>#4</td>
<td>C + P + ß (2 mL)</td>
<td>Water (5 mL)</td>
<td>Water (5 mL)</td>
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<tr>
<td>#5</td>
<td>Buffer (2 mL)</td>
<td>50% ethanol (5 mL)</td>
<td>50% ethanol (5 mL)</td>
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<tr>
<td>#6</td>
<td>C + P + ß (2 mL)</td>
<td>50% ethanol (5 mL)</td>
<td>50% ethanol (5 mL)</td>
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<td>#7</td>
<td>50% ethanol (5 mL)</td>
<td>Buffer (2 mL)</td>
<td>50% ethanol (5 mL)</td>
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<td>#8</td>
<td>50% ethanol (5 mL)</td>
<td>C + P + ß (2 mL)</td>
<td>50% ethanol (5 mL)</td>
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<tr>
<td>#9</td>
<td>Water (5 mL)</td>
<td>Buffer (2 mL)</td>
<td>50% ethanol (5 mL)</td>
</tr>
<tr>
<td>#10</td>
<td>Water (5 mL)</td>
<td>C + P + ß (2 mL)</td>
<td>50% ethanol (5 mL)</td>
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</table>

* Samples using solvents at 2 mL were extracted in a 50°C water bath, while those using 5 mL were extracted at room temperature.

* 0.2 M sodium acetate buffer (pH 4.8).

* Mixture of cellulase (C), pectinase (P) and β-glucosidase (ß) in a final volume of 2 mL. 0.2 M sodium acetate buffer (pH 4.8).
To investigate enzyme activities at pH 4.8 and 50 °C (data not shown). After 1 h of shaking, the samples were centrifuged at 1500 rcf in an Eppendorf Centrifuge 5702 (Brinkmann Instruments Inc., NY, USA) for 3 min, and the resulting supernatants collected, filtered through a Whatman #4 filter paper (0.45 μm) and used as Extraction 1 samples. The residue was re-extracted two more times following Table 1 protocols to obtain Extraction 2 and Extraction 3 samples. All supernatants were stored at -20°C until analyzed. All samples from Extractions 1, 2 and 3 were analyzed in duplicate for total phenolics and the Noble skin samples for anthocyanins.

2.2.2.1. Enzyme type and incubation time. To investigate enzyme type and incubation time, 0.25 g of freeze-dried and ground skins or seeds were weighed into separate tubes and extracted with 2 mL selected solvents (50% ethanol, buffer, cellulase, pectinase, β-glucosidase, and a mixture of these enzymes) for 1, 4, 8, and 24 h at 50°C. Ethanol (50%) was used as control. The enzyme concentrations were previously described. After 1 h of shaking, the samples were centrifuged at 1500 rcf for 3 min, and the resulting supernatants were collected, filtered (0.45 μm) and used as Extraction 1 samples. The residue remaining after enzyme hydrolysis was collected and subjected to a subsequent extraction at room temperature for 1 h by adding 5 mL 50% ethanol, shaking and repeating the centrifugation. This supernatant was collected, filtered (0.45 μm) and used as Extraction 2 samples. All supernatants were stored at -20 °C until analyzed. All extractions (Extraction 1 and 2) were analyzed for total phenolics and antioxidant activity in duplicate.

2.2.2.2. Analysis of total phenolics. Total phenolic content in grape seeds or skins was determined by the method of Singleton and Rossi (1965) using an ultraviolet–visible Beckman Coulter DU-640 spectrophotometer (Beckman Instruments, CA, USA). A mixture of 100 μL 5-fold diluted extract, 3900 μL distilled water, 250 μL of Folin–Ciocalteau reagent (2 N), and 750 μL of 20% Na2CO3 were introduced in a tube. After reacting for 30 min in a 40 °C water bath, absorbance was measured at 760 nm. Gallic acid (GA) was used as standard and expressed as gallic acid equivalents (mg gallic acid (GAE)/g dry matter (DM)) using a calibration curve. The linearity range of the calibration curve was 100–800 μg/mL (R² = 0.9996).

2.2.2.3. Identification of individual phenolics. The obtained extracts were subjected to chromatographic analyses on a Hitachi HPLC system with a Zorbax Stablebond Analytical SB-C18 column (4.6 mm, 250 mm, 5 μm, Agilent Technologies). Elution was performed using mobile phase A (0.5% formic acid aqueous solution) and mobile phase B (60% methanol with 0.5% formic acid). The following linear gradient was used: 0–5 min: 5% B, 5–10 min: 15% B, 10–20 min: 25% B, 20–30 min: 50% B, 30–40 min: 70% B, 40–50 min: 90% B. The flow rate was 0.9 mL/min. Injection volume was 20 μL with the UV detector set to an absorbance wavelength of 280 nm. The retention times for each of the different components in the analyzed pool were compared to the following standards: gallic acid, catechin, epicatechin, epicatechin gallate, trans-resveratrol, ellagic acid, and quercetin.

Standards for pentagalloyl glucose, ellagic acid hexoside and monogalloyl and digalloyl glucose were not available. Thus, a second HPLC system was employed to confirm the identification of these by ESI–MS detection in the Selective reaction monitoring (SRM) mode. The LC–MS analyses were performed on a Thermo-Finnigan Surveyor HPLC system (Thermo-Finnigan, St. Jose, CA, USA) equipped with a TSQuantum controlled by Xcalibur data analysis software (version 1.3, Thermo-Finnigan). The column and mobile phase used were the same as described above in HPLC analysis. The MS acquisition was with ESI interface in negative ionization mode at the following conditions: sheath gas (N2) pressure, 42 arb; auxiliary gas (N2) pressure, 20 arb; spray voltage 3.96 kV; capillary temperature, 414 °C; collision gas pressure, 1.5 mTorr; collision energy, 22 V. These compounds were identified on the basis of their mass fragmentation data compared with and identical to those reported by Sandhu and Gu (2010).

2.2.2.4. Analysis of antioxidant activities by DPPH Assay. The DPPH assay was based on the slightly modified method of Brandwiliams, Cuvelier, and Berset (1995). The 10-fold diluted sample (100 μL) was added to 3.9 mL methanolic solution of DPPH (0.0025 g/100 mL CH3OH). After 60 min reaction at room temperature in the dark, the absorbance at 515 nm was recorded to determine the concentration of the remaining DPPH. The percentage inhibition of DPPH in the test sample and known concentrations of Trolox were calculated by the following formula: %Inhibition = 100 × (A0 – A)/A0, where A0 was the beginning absorbance at 515 nm, obtained by measuring the same volume of solvent, and A was the final absorbance of the test sample at 515 nm. The calibration curve between %Inhibition and known concentration of Trolox solutions was then established. The radical scavenging activities of the test samples were expressed as Trolox equivalent antioxidant capacity (μmol TE/g DM) from their percentage inhibition. Trolox standard solutions were prepared at a concentration ranging from 100–1000 μmol (R² = 0.9999).

2.2.3. Ratio of antioxidant activity to total phenolics

The following Noble seed extracts were selected in this test: (1) samples extracted with 50% ethanol for 8 h – Extraction 1 (EtOH-8h-1), and (2) the resulting residue further extracted with 50% ethanol for 1 h – Extraction 2 (EtOH-1h-2); (3) samples extracted with a mixture of cellulase, pectinase and β-glucosidase in buffer for 8 h – Extraction 1 (Enzyme-8h-1), and (4) the resulting residue further extracted with 50% ethanol for 1 h – Extraction 2 (Enzyme-1h-2) (Table 2). Each extract was separated by HPLC and fractions were collected every 5 min, starting around 3 min when the first peak appeared and terminated after 43 min. Eight fractions were then subjected to total antioxidant activity and phenolic assay, and ratio of antioxidant activities (DPPH values) to total phenolics was expressed as mmol Trolox per gram Gallic acid.

To further investigate enzyme incubation treatment on grape phenolics, Noble seed samples: EtOH-4h-1 (extracted with 50%
ethanol for 4 h – Extraction 1) and EtOH-8h-1 were dried under nitrogen and then incubated with the mixture of cellulase, pectinase and β-glucosidase in 2 mL buffer for 4 or 8 h at 50 °C. EtOH-4h-1, EtOH-8h-1 and their hydrolyzed samples were separated by HPLC and fractions were collected and analyzed as previously described.

2.3. Statistical analyses

All extractions and analyses were performed in duplicate. Data were subjected to ANOVA and differences among samples were tested by post hoc comparison test (Student Newman Keuls) at p = 0.05 with Microsoft Excel 2008 and SPSS 16.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Different combinations of solvent or enzyme on release of phenolics from grape skin and seed

Total phenolics released by the 10 combinations of solvent or enzyme for Noble and Carlos skins showed significant differences while those from seeds showed slight difference (Fig. 1a, c, d), since patterns for Noble were similar, while the Carlos data was presented. All first extractions released a majority of the total phenolics. The 50% ethanol (#2) was the most effective solvent for extracting total phenolics from grape skins and seeds, followed by combinations of enzyme and solvent (#3–10) while the least effective was water (#1). For Noble skins, combinations of buffer (enzymes) and 50% ethanol (#5, 6, 7, 8) yielded the highest total phenolics (average 43.38 mg GA/g DM) comparable to the water control (#1). For Carlos skins, 50% ethanol-buffer (enzymes)-50% ethanol combinations (#7, 8) yielded the highest total phenolics (average 50.20 mg GA/g DM) comparable to the water control (#1). Extracting first with buffer or enzymes (#3, 5) significantly decreased the total phenolic yield compared to no enzyme treatments (#3, 5). Further, the 50% ethanol control (#2), and significantly higher by 45% than the water control (#1). For Noble skins, combinations of buffer (enzymes) and 50% ethanol (#5, 6, 7, 8) yielded the highest total phenolics (average 34.83 mg GA/g DM) compared to the water control (#1). For Carlos skins, 50% ethanol-buffer (enzymes)-50% ethanol combinations (#7, 8) yielded the highest total phenolics (average 34.83 mg GA/g DM) comparable to the water control (#1). Extracting first with buffer or enzymes (#3, 5) significantly decreased the total phenolic yield compared to their second extraction with buffer or enzymes (#7, 8). Furthermore, enzyme hydrolysis used in Extraction 1 (#4, 6) reduced the release of phenolics compared to no enzyme treatments (#3, 5). Phenolic extraction from Noble and Carlos grape seeds were similar to Carlos skin with 50% ethanol-buffer (enzymes)-50% ethanol combinations (#7, 8), and 50% ethanol control (#2) yielding
highest total phenolics (about 70 mg GA/g DM) while the water control (#1) yielded the lowest total phenolics (about 35 mg GA/g DM).

When compared with combinations of buffer and solvent (#3, 5, 7, 9), combinations of enzyme and solvent (#4, 6, 8, 10) did not result in an enhanced release of total phenolics from the studied skins and seeds (Fig. 1). This finding was consistent with the report of Li et al. (2006) that citrus peel phenolics released by enzymes is in fact due to water, and especially hot water. In contrast, enzyme treatments severely depressed the yield of total phenolics in Carlos skin (#4 vs. #3, #6 vs. #5). Further studies showed that this was caused by cellulase (Table 2). It was probably cellulase hydrolysis that caused disorder in the Carlos skin cell wall so as to retard phenolics extracted by solvents in Extraction 2. However, cellulase did not severely depress the yield of total phenolics in Noble skin. This was possible because the major phenolics in Noble skin were anthocyanins (in Carlos skins, they are non-anthocyanins). Anthocyanins are water-soluble vacuolar pigments, not bound to the grape skin polysaccharide matrix, and are instantly released from the skin during the early phase of the enzymatic treatments (Arnous & Meyer, 2010).

Fig. 1(b) showed that treatments #4 and 6 in Noble skins yielded the highest total anthocyanins (average 23.15 mg CAE/g DM), which was 28% higher than the water control (#1) and 10% higher than the 50% ethanol control (#2). Thus, the release of total phenolics in Noble skin was not severely affected by enzyme treatments #4 and #6 while it was for Carlos skin.

Enzymes or buffer used in Extraction 2 (#7, 8) yielded similar total phenolics compared to the 50% ethanol control (#2), and significantly higher levels than Extraction 1 (#5, 6). This was because most of the phenolics in treatments #7 and 8, as well as #2, were released in Extraction 1 by the 50% ethanol, and not influenced by the addition of enzyme in Extraction 2. Most often, enzyme hydrolysis used for phenolic extraction was performed initially and then extracted with other solvents (Chamorro et al., 2012; Kammerer et al., 2005; Meyer, Jepsen, & Sorensen, 1998). However, our results showed that treating muscadine grape skins and seeds this way decreased the total phenolic yields.

3.2. Enzyme type and incubation time on release of phenolics from grape skin and seed

Previously, Lee and Talcott (2005) studied hydrolysis of α-gluco- sidease and tannase on ellagic acid derivatives in extracts from muscadine grapes (Vitis rotundifolia Michx.) and found that α-glucosidase could effectively hydrolyze ellagic acid glycosides whereas tannase had little effect on ellagic acid precursors. In this study, three different enzymes (cellulase, pectinase, and β-glucosidase) and incubation times were investigated as to their release of grape phenolics. Thus, muscadine grape skins and seeds were subjected to individual or mixed enzyme hydrolysis for different times followed by extraction with 50% ethanol.

3.2.1. Noble skins

For enzyme Extraction 1, compared to the yield from buffer, cellulase hydrolysis had no significant influence on increasing the release of total phenolics (Table 2). Cellulase hydrolysis increased phenolic release as incubation time increased (1–8 h) but this was more related to the extension of incubation time than enzyme. However, longer incubation time (24 h) decreased the total phenolic yield. This was probably due to temperature (50°C) and additional side reactions during the long hydrolysis time (Arnous & Meyer, 2010; Chen & Lin, 2007). During the first hour of incubation,
ß-glucosidase hydrolysis significantly increased the release of total phenolics by 12%. However, longer incubation with ß-glucosidase showed no additional benefit compared to buffer control. Pectinase was better than cellulase and ß-glucosidase and significantly increased (by 72%) total phenolics at first (1 h), but then the total phenolic yield decreased with longer incubation time. It is possible that pectinase helps break down the structural polysaccharides of the cell wall quickly, thereby accelerating the release of phenolics; however, all released phenolics then become exposed to the adverse conditions (temperature, cell enzymes, etc.) during the long incubation time and start to degrade (Arnous & Meyer, 2009). Hydrolyzing with the mixture of enzymes also significantly increased the release of total phenolics during the first 4 h of incubation, but the yield decreased after 24 h hydrolysis. This was mainly driven by pectinase while there was little contribution from cellulase and ß-glucosidase. Even though the phenolics yield was lower than the 50% ethanol control, enzyme hydrolysis did enhance or accelerate to some extent the release of total phenolics in Noble skins. Phenolics extracted by 50% ethanol almost reached maximum yield after 1 h extraction and were stable till 8 h, but beyond this time, the yield in total phenolics significantly decreased at 24 h hydrolysis. A possible reason was the long incubation time at a high temperature (50°C) degraded the phenolics (Chen & Lin, 2007).

Since the enzyme extraction was performed in buffer, an appreciable amount of non-water-soluble phenolics such as highly polymerized procyanidins may be retained in the residue. To recover these phenolics, Extraction 2 was performed with 50% ethanol for 1 h. Table 2 shows that as hydrolysis time for Extraction 1 increased, phenolic recovery in Extraction 2 for the 50% ethanol control increased, especially for the 24 h treatment. This may be the result of the precipitated phenolics in the residue re-dissolving in the solvent. For Extraction 2, more phenolics (about 40–60%) were recovered after enzyme treatment than the 50% ethanol control, especially the early incubation times.

By combining the two extractions (1 and 2), enzyme treatment yielded about 90% of the phenolics from the 50% ethanol control (Table 2), similar to the rate of 87.9% reported by Li et al. (2006). However, when compared with buffer, enzyme hydrolysis seemed to only accelerate the release of phenolics rather than enhance the release of total phenolics in Noble skins.

The release of Noble skin anthocyanins by enzymes was similar to the release of their total phenolics (Table 2) and probably related to anthocyanins being the major phenolics in Noble skins. Cellulase hydrolysis slightly decreased the release of total anthocyanins while ß-glucosidase and pectinase hydrolysis significantly increased (by 13% and 70%, respectively) the release of total anthocyanins at the early hydrolysis time (1 h). The effect of pectinase was consistent with the reports of Kammerer et al. (2005) and Haight and Gump (1994) while the outcome of cellulase was not. ß-glucosidase was reported to degrade anthocyanins by deglycosylation and formation of unstable anthocyanin aglycones (Wrolstad et al., 1994). However, in this study, this seems to have taken place after 4 h hydrolysis in view of the significant decrease in anthocyanins after this time.

A previous report (Lee & Talcott, 2005) showed that α-glucosidase could effectively hydrolyze phenolic glycoside compounds of muscadine grapes. Therefore, ß-glucosidase was used in this study to investigate whether it had the same consequence on the released muscadine grape skin and seed polyphenols. Results suggested that ß-glucosidase also could effectively hydrolyze phenolic glycoside compounds of muscadine grapes (such as pentagalloyl glucose), and release low molecular weight phenolics with high antioxidant activity (such as gallic acid) (Fig. 2). The use of ß-glucosidase also confirmed the previous report (Wrolstad et al., 1994) that it could degrade anthocyanins, but in this study the degradation of anthocyanins was not immediate. Major degradation took place after 4 h hydrolysis (Table 2). This was consistent with Arnous and Meyer's (2010) work that the enzyme catalyzed decay of the acylated anthocyanins most likely took place via a sequential enzyme catalyzed degradation caused as both cinnamate esterase activity and ß-glucosidase activity: as a first step, cinnamate esterase catalyzes the stripping of the acylated anthocyanins from its shielding hydroxyccinnamic acid (p-coumaric, caffeic, ferulic acids), which makes it possible for ß-glucosidase to deglycosylate anthocyanins releasing glucose and the more susceptible aglycone form of anthocyanins.

3.2.2. Carlos skins

Enzyme release of phenolics in Carlos skins was significantly different from Noble skins (Table 2). For enzyme Extraction 1, cellulase hydrolysis severely reduced (by 50%) the release of total phenolics in Carlos skins, even though the released phenolics increased slightly with increasing hydrolysis time. Pectinase or ß-glucosidase at 1 h hydrolysis time increased (by 11%) the release of total phenolics in Carlos skins. However, with the increase in hydrolysis time, the release of total phenolics decreased. Hydrolysis at 1 h for the mixture of enzymes severely inhibited (by 34%) the release of total phenolics in Carlos skins, and with the increase in incubation time, the release of total phenolics decreased. This effect was mainly driven by cellulase while very little contribution occurred from pectinase or ß-glucosidase. Compared to the 50% ethanol control, enzyme hydrolysis released only half or less total phenolics from Carlos skins. For Extraction 2, Carlos skins showed a similar trend as Noble skins, that is, about 40–60% phenolics were recovered from enzyme treatments compared to the 50% ethanol control, and the recovered phenolics decreased with increasing incubation time. When adding the two extractions (1 and 2) together, enzyme treatments yielded 48–65% of the phenolics extracted by the 50% ethanol control. When compared with buffer, enzyme hydrolysis had very little or no influence on the release of phenolics in Carlos skins.

3.2.3. Noble and Carlos seeds

Enzyme release of phenolics in Noble and Carlos seeds was similar (Table 2). For enzyme Extraction 1, enzyme hydrolysis showed little influence on the release of total phenolics. This is because the cell wall of grape seeds is hard and very resistant to chemical and biological degradation (Düsterhoff, Engels, & Voragen, 1993; Xu, Zhang, Wang, et al., 2010). The maximum phenolic yield in Noble seeds was obtained at 4 h incubation while in Carlos seeds it took 8 h. Compared with the 50% ethanol control; enzyme hydrolysis only released about 35% of the total phenolics from Noble seeds and 48% from Carlos seeds. Besides the degradation issue of the grape seed cell wall, another factor for the low yield is that the major phenolics are highly polymerized procyanidins and are non-water-soluble. For Extraction 2, about 50–60% phenolics were recovered from enzyme treatments, and the recovered phenolics decreased with increasing hydrolysis time. For the two extractions (1 and 2), enzyme treatments yielded 70–80% phenolics when compared to the 50% ethanol control. Enzyme hydrolysis after 1 h yielded the maximum phenolics and the amount decreased with increasing hydrolysis time.

3.3. Enzyme type and hydrolysis time on antioxidant activities from grape skin and seeds

The DPPH method which has been extensively used to measure the free-radical-scavenging activity of pure antioxidant compounds extracted from fruits, plants, or food materials was used in this study to characterize the total antioxidant activity. Enzyme type and hydrolysis time on grape skin and seed antioxidant...
activities were very similar to the patterns seen for their total phenolics (Table 3). This is in agreement with previous reports that antioxidant activities of grapes have good correlation with their phenolic content (Xu, Zhang, Zhu, Huang, & Lu, 2011). Although...
The increase in level of phenolic acids after enzyme treatment indicated that the use of pectinolytic and cellulolytic enzymes in grape pomace (V. vinifera) decreased the yield of gallic acid.

To further explain the increase in antioxidant activities of grape phenolics after enzyme hydrolysis, several Noble seed samples were separated into eight fractions by HPLC, and then the ratio of antioxidant activity to total phenolics for each fraction was tested. Fig. 2 shows the chromatographic profiles of phenolics released by 50% ethanol (a, b) and enzymes (c, d). Low molecular weight phenolics, especially gallic acid, mono- and di-galloyl glucose, epicatechin, and ellagic acid and its conjugates were the major phenolics present after enzyme hydrolysis; while phenolics extracted by the 50% ethanol treatment had more galloylated forms, such as epicatechin gallate and pentagalloyl glucose. This indicated that enzyme hydrolysis changed the galloylated form of muscadine grape (V. rotundifolia) phenolics (epicatechin gallate and pentagalloyl glucose) to low molecular weight phenolics, releasing phenolic acids such as gallic acid and mono- and di-galloyl glucose. This finding was consistent with the report of Chamorroa et al. (2012) that the use of tannase and pectinase in grape pomace (V. vinifera) changed the galloylated form of epicatechin to its free form, releasing gallic acid and increasing the antioxidant activity; however, it contradicted the report by Kammerer et al. (2005) that the use of pectinolytic and cellulolytic enzymes in grape pomace (V. vinifera) decreased the yield of gallic acid.
fraction 2 (15.6 mmol/g) and EtOH-8h-1-Enzyme fraction 2 (14.5 mmol/g) were significantly higher than EtOH-4h-1 and EtOH-8h-1 fractions, which ranged from 6.4 mmol/g to 13.3 mmol/g (Fig. 3b). This demonstrated that enzyme hydrolysis could modify released phenolics to more potent antioxidant compounds.

4. Conclusions

The results obtained in this study demonstrated that combinations of solvent and enzyme, and treatment order greatly affected the release of phenolics from muscadine grape skins and seeds. Pre-treating muscadine skin and seed samples with enzymes, which is the most common method of hydrolysis, decreased total phenolic yield compared with solvent (50% ethanol) alone. For different muscadine varieties, enzymatic release of phenolics from skins was significantly different while for seeds, they were similar. One hour pectinase hydrolysis significantly increased the release of total phenolics in Noble skins while no apparent outcome was observed for cellulase and β-glucosidase hydrolysis; cellulase hydrolysis severely inhibited the release of total phenolics in Carlos skins while pectinase or β-glucosidase hydrolysis increased the release of total phenolics. Therefore, both enzyme type and grape varieties should be considered to achieve an effective enzyme method for releasing phenolics from grape skins. Since adverse conditions (temperature, cell enzymes, etc.) could degrade phenolics affecting their total yield, long incubation times should be avoided. Enzyme hydrolysis was found to shorten extraction time. Most importantly, enzyme hydrolysis could modify the galloylated form of phenolics to low molecular weight phenolics, releasing higher antioxidant

![Fig. 3. The ratio of antioxidant activities (DPPH values) to total phenols for different fractions of Noble seeds by various treatments.](image-url)
activity in the form of phenolic acids, especially gallic acid. Further studies are needed to explore the specific pathway of enzyme modification for phenolics to their more potent antioxidant compounds. This will help convert the complex polyphenols into their more potent antioxidant compounds. For this reason, enzyme release of phenolics might have applicability in the food and nutraceutical industries.

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References


