

Polyphenolic Contents and Antioxidant Activities of Underutilized Grape (*Vitis vinifera* L.) Pomace Extracts

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ABSTRACT: Grape pomace is an abundant source of underutilized winery by-products. Polyphenols were extracted from grape pomace using cellulase and gluco-amylase enzymes. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocalteu's assays were used to measure antioxidant activity and total polyphenolic contents. Both cellulase, and gluco-amylase digested grape pomace showed efficient radical scavenging activity. In addition, the total polyphenolic contents of cellulase digested grape pomace showed lower concentrations were effective compared to higher concentrations, whereas gluco-amylase enzyme did not show remarkable variations. The DPPH radical scavenging activity and total polyphenolic contents were significantly higher in the cellulase digested grape pomace compared to the gluco-amylase digested and the not digested grape pomace. It is notable that enzymatic digestions were efficient for extracting polyphenols from grape pomace. The underutilized grape pomace polyphenols can be further used for food safety as a natural antioxidant.

Keywords: grape pomace, polyphenols, antioxidant activity

INTRODUCTION

The world grape (*Vitis vinifera* L.) production is approximately 60 million tons per annum (1). Every year about 80% of the world grapes are utilized for winemaking and about 20% are waste by-products (2). Grape pomace contains polyphenols having useful bioactive components. The major polyphenolic compounds found in grape wastes are anthocyanins, catechins, glycosides of flavonols, and polyphenolic acids (3). Grape skins are rich sources of anthocyanins, hydroxycinnamic acids, flavanols, and flavonol glycosides; flavanols are mainly present in the seeds (4). Anthocyanins, catechins, flavonol glycosides, polyphenolic acids, alcohols, and stilbenes are the principal polyphenolic compounds in grape pomace (5). Blackberry, blueberry and red wine grapes show remarkable variations in total anthocyanins, total flavonols as well as antioxidant activities (6). Polyphenolic compounds, as important plant constituents, exhibit antioxidant activities by inactivating lipid free radicals, or preventing decomposition of hydroperoxides into free radicals (7). Grape wastes can be used for extraction of polyphenols and be used as a source of antioxidants (8). Flavonoids are effective antioxidant poly-

phenolic compounds (9). Grape pomace is a valuable source of polyphenolic compounds, which could be utilized as functional food components. The Folin-Ciocalteu's method is widely-used to assess total polyphenolic contents, although different polyphenolic compounds show different responses in the Folin-Ciocalteu's assay (10). Polyphenols, as secondary plant metabolites, play a critical role in human health and are nutritionally important (11). Epidemiological studies indicate that fruits, vegetables and plant-based polyphenolic metabolites are beneficial to human health due to their potent antioxidant, anticancer and platelet aggregation inhibitory activities (12). Grape pomace has great potential as a source of total polyphenolic compounds with high antioxidant activity. Solubility of polyphenolic compounds depends mainly on the hydroxyl groups, the molecular size, and the length of hydrocarbons. Reduction of particle size increases the rate of extraction of polyphenols as well as the extraction yield. The process can be enhanced by grinding and by enzymatic treatment. Solvent and process are the variables on polyphenols extraction. Extraction of polyphenols by enzymatic treatment can be suitable to maximize the antioxidant yields and activities. It was observed that the extraction yield was in-

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creased by the action of pectinases, cellulases and hemicellulases (13-15). Grape pomace is generally underutilized and discarded by the wine industry as waste by-products. No systematic method has been established to utilize grape pomace polyphenols. An effective method for extracting grape pomace polyphenols is an ongoing demand as it is a good source of natural bioactive compounds. Here, we made an attempt to treat the samples with enzymatic digestion using cellulase and gluco-amylase to ease the extraction of polyphenols from grape pomace as well as to assess its antioxidant activity.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu's reagent, ethanol (ethyl Alcohol 99.5%), sodium carbonate anhydrous (Na_2CO_3), and Tris-HCl (2-amino-2-hydroxymethyl-1,3-propanediol) were purchased from WAKO Pure Chemical Industries Ltd. (Tokyo, Japan). Chlorogenic acid (3-caffeoylquinic acid) was purchased from MP Biomedicals (Santa Ana, CA, USA). DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cellulase and gluco-amylase enzymes were obtained from Yakult Chemical Industries Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Extraction

Grape pomace was collected from the Nagano, Japan. Koshu grapes were used for wine making and the grape pomace (winery by-products) was collected in the year of 2012 for extraction of polyphenols. Ten grams of samples were weighed using an electronic balance and 90 mL of phosphate buffer saline (PBS), pH 7.3 was added. Then the samples were homogenized using a blending machine. After homogenization, samples were digested using the enzymes. Cellulase and gluco-amylase enzymes of various concentrations (0.25, 0.5, 1.0, and 2.5 mg/mL) were added to the homogenized samples, and incubated at 55°C for 24 h. Enzyme solutions were prepared using sodium citrate buffer (pH 4.2). The samples were divided into cellulase digested, gluco-amylase digested, and non-digested group. After enzymatic digestion the samples were centrifuged at 5,000 rpm for 20 min and the supernatants were collected. The extracted supernatants were filtered through Whatman no. 4 filter paper to remove unutilized residues. Supernatants were vacuum dried to remove organic solvents and then lyophilized for the collection of samples. Fig. 1 shows the procedure of preparation of grape pomace extracts (GPE).

Folin-Ciocalteu's assay

The total polyphenolic contents were measured using the Folin-Ciocalteu's assay, a slight modification of the Singleton method (16). Ten μL /well of sample solution of different concentrations, or standard solution, were added into a 96-well microplate, followed by 40 μL /well of ion exchanged water, and 50 μL /well of a mixture containing Folin-Ciocalteu's reagent and ion exchanged water (1:7 v/v). After 5 min at room temperature, 50 μL /well of 9% sodium carbonate aqueous solutions was added. The mixture was allowed to stand for 90 min at room temperature in a dark place. The absorbance was read at 595 nm using a microplate reader (Bio-Rad Model 680 microplate reader, Bio-Rad Laboratories Inc., Hercules, CA, USA). Total polyphenolic contents were expressed as chlorogenic acid equivalents (ChAE) per gram dry weight of extracted samples. A standard curve was prepared to express the results as chlorogenic acid equivalents, i.e. the quantity of chlorogenic acid (mg/mL).

DPPH assay

DPPH was used to evaluate the free radical scavenging activity. The sample solutions were prepared by dilution of the extracted samples with PBS, pH 7.3. The reaction was initiated by addition of 100 μL /well DPPH solution in ethanol (60 $\mu\text{g}/\text{mL}$), or ethanol for color control. The DPPH solutions were prepared daily, covered with aluminum foil, and kept in a dark place at 4°C between measurements. The reaction mixture was incubated at room temperature for 30 min in a dark place and absorbance was measured at 517 nm following the method of Yu et al. (17) with slight modifications. The absorbance was measured using a microplate reader (Bio-Rad Model 680 microplate reader, Bio-Rad Laboratories Inc.). The DPPH radical scavenging activity was expressed as %

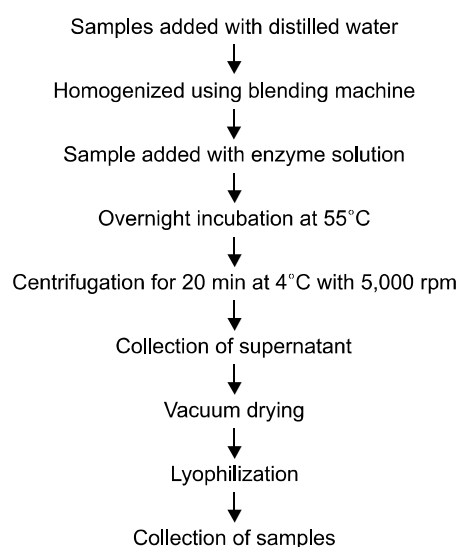


Fig. 1. Flow diagram for extraction of grape pomace.

DPPH inhibition/mg of GPE. Inhibition of sample treatment was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{A_0 - A}{A_0} \times 100$$

where A_0 is absorbance control and A is absorbance sample.

Statistical analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test (DMRT). Statistical probability $P < 0.05$ was considered significant. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Total polyphenols

The total polyphenolic contents were expressed as mg/g dry weight (DW) of grape pomace, following a standard curve of chlorogenic acid as ChAE. Fig. 1 shows the flow diagram of extraction of grape pomace polyphenols. As shown in Fig. 2, grape pomace extracts contain significant ($P < 0.05$) amounts of polyphenols depending on the extraction process. Not digested (ND), and both cellulase digested (CD) and gluco-amylase digestion (GD) showed variations in polyphenolic contents. The polyphenolic contents of CD were 41.05 ± 1.07 , 40.54 ± 0.49 , 39.64 ± 0.48 , and 39.19 ± 0.31 mg ChAE/g, respectively, against 0.25, 0.5, 1.0, and 2.5 mg/mL enzyme concentrations. In addition, the polyphenolic contents of GD were 33.42 ± 0.24 , 33.36 ± 0.29 , 33.25 ± 0.39 , and 33.19 ± 0.28 mg ChAE/g, respectively, against 0.25, 0.5, 1.0, and 2.5 mg/mL enzyme concentrations, and the polyphenolic contents of ND was 32.29 ± 0.84 mg ChAE/g without

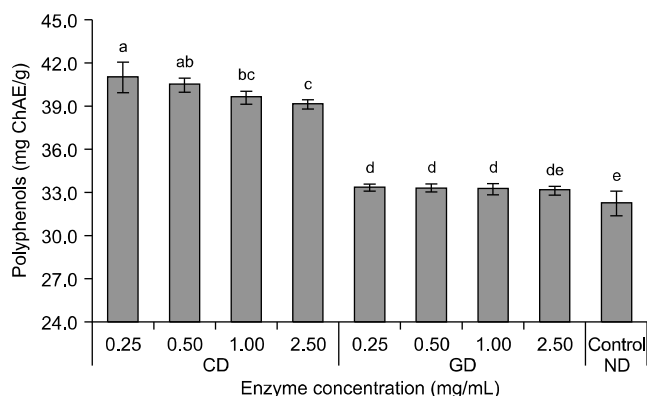


Fig. 2. Total polyphenolic contents of grape pomace (mg ChAE/g). CD, cellulase digested; GD, gluco-amylase digested; ND, not-digested. Enzyme concentrations were 0.25, 0.5, 1.0, and 2.5 mg/mL. Each value represents mean \pm SD ($n=3$). Different letters (a-e) indicate significant differences ($P < 0.05$).

addition of enzyme. The polyphenolic contents of the ethanolic, methanolic, and water extracts of grape pomace were 27.9, 35.7, and 6.1 (w/w), respectively (18). An 80% ethanolic extract of grape pomace contained 30.4 mg gallic acid equivalent (GAE)/g of total polyphenolic contents (19). Polyphenolic contents after enzymatic digestion varied depending upon enzyme concentrations. Enzyme concentrations of 0.25 and 0.50 mg/mL showed significantly ($P < 0.05$) higher polyphenolic contents compared to 1.0 and 2.5 mg/mL, whereas no significant differences were shown between 0.25 and 0.50 mg/mL. Both cellulase digested and gluco-amylase digested enzyme groups showed significantly ($P < 0.05$) higher polyphenolic contents than ND. In addition, the total polyphenolic contents in cellulase digested grape pomace were negatively associated with the concentration of cellulase, while the gluco-amylase digested group showed nearly no change. In Carménère and Cabernet Sauvignon grape seeds, the total polyphenolic contents of ethanolic extracts (1:9 v/v ethanol/water) varied from 21.8 to 16.6 mg GAE/g and 20.4 to 17.5 mg GAE/g, respectively, depending on harvest time (20). High concentrations of polyphenolic compounds were found in Brazilian grape seeds ranging from 2,128 to 16,518 mg and in the skins ranging from 660 to 1,839 mg of catechin equivalents (CE/100 g) (21). Grape pomace contains varied amounts of polyphenolic compounds (22). Grape pomace polyphenols differ significantly, depending mainly on the cultivar and vintage, and on the degree of ripeness and the technology applied during vinification (5).

DPPH radical scavenging activity

Polyphenolic compounds have been recognized as important sources of natural antioxidants. Fig. 3 shows the DPPH radical scavenging activity of grape pomace extracts. Grape pomace is a potential source of natural

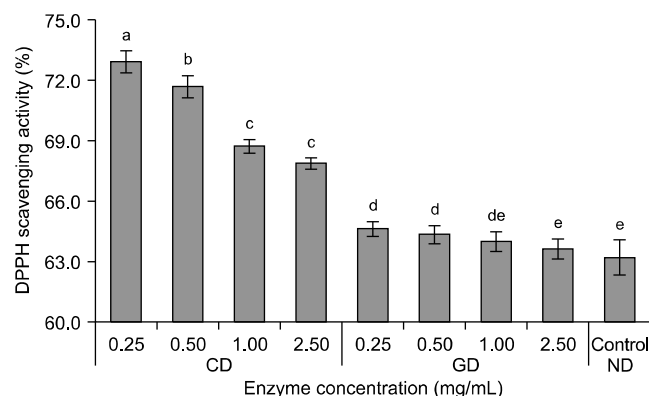


Fig. 3. DPPH radical scavenging activity of grape pomace polyphenols. CD, cellulase digested; GD, glucoamylase digested; ND, not-digested. Enzyme concentrations were 0.25, 0.5, 1.0, and 2.5 mg/mL. Data represents mean \pm SD ($n=3$). Different letters (a-e) indicate significant differences ($P < 0.05$).

antioxidants. Both cellulase enzyme digested and gluco-amylase enzyme digested grape pomace extracts showed significantly ($P < 0.05$) higher antioxidant activities compared to the control. Grape pomace extracts showed varied amounts of DPPH radical scavenging activities (23). Ethanol extracts exhibited the highest antioxidant activity compared to other solvent extracts, to synthetic food antioxidants BHT, ascorbyl palmitate, and to the natural food antioxidant, vitamin E, and no correlation was found between antioxidant activity and total polyphenolic contents (24). Enzymatic digestion showed marked variations on the DPPH radical scavenging activity of grape pomace polyphenols extracts. The DPPH radical scavenging activities also varied depending on enzyme concentrations. The DPPH radical scavenging activity of CD were $72.96 \pm 0.55\%$, $71.71 \pm 0.56\%$, $68.74 \pm 0.32\%$, and $67.91 \pm 0.29\%$, respectively, against 0.25, 0.5, 1.0, and 2.5 mg/mL enzyme concentrations. In contrast, the DPPH radical scavenging activities of GD were $64.63 \pm 0.35\%$, $64.33 \pm 0.47\%$, $63.99 \pm 0.50\%$, and $63.64 \pm 0.48\%$, respectively, against 0.25, 0.5, 1.0, and 2.5 mg/mL enzyme concentrations, and the DPPH radical scavenging activity of ND was $63.21 \pm 0.88\%$ without addition of enzyme. The enzyme concentration of 0.25 mg/mL showed significantly ($P < 0.05$) higher DPPH radical scavenging activity, irrespective of enzyme digested groups. Both enzyme groups showed significantly ($P < 0.05$) higher DPPH radical scavenging activity than the control (ND). In the cellulase digested group, enzyme concentrations showed significant variations in DPPH radical scavenging activity, while enzyme concentrations did not show marked variations in the gluco-amylase digested group. The ethanolic, methanolic, and water extracts of grape pomace exhibited 42.1, 67.3, and 9.1% free radical scavenging activities, respectively, at 50 ppm (18).

Grape pomace is a potential source of polyphenols and natural antioxidants. Cellulase digested grape pomace showed significantly higher polyphenolic contents in the Folin-Ciocalteu's assay, and it also showed significantly higher reductive activities in DPPH radicals, compared to the gluco-amylase digested and the not digested groups. The enzyme extracted polyphenols from underutilized grape pomace can be further utilized in the food industry during food manufacturing as a source of natural antioxidants.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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