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Effective Extraction of Oligomeric Proanthocyanidin (OPC) from Wild Grape Seeds

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Abstract The Oligomeric proanthocyanidin (OPC) in green and black tea, grape seeds, grapes and wine has raised much attention but that OPC in wild grape seed remains to be intensively investigated. This study investigated the total OPC contents and total antioxidant activity of wild grape seeds and developed an efficient extraction process with various temperatures, solvent compositions and times. Also, a chromatography column packed with the Dia-ion HP-20 resin was used for further purification of the OPC. The total OPC contents were determined with the Folin-Ciocalteu reagent, and the antioxidant activity using total antioxidant potential (TAP) and 1,1-diphenyl-2picrylhydrazyl (DPPH). The yield of final purified OPC was 1.78 (+)-catechin equivalent (CE) g/100 g, with IC_{50} activities of TAP and DPPH of 31.60 and 15.70 μg/mL. These activities of the final purified OPC were about two times higher than that of the BHA used as a reference sample.

Keywords: wild grape seed, oligomeric proanthocyanidin (OPC), antioxidant activity, DPPH

INTRODUCTION

Free radicals have been implicated in over a hundred human disease conditions in humans, including arthritis, hemorrhagic shock, and advancing age [1]. Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic processes [2]. A large number of chemical antioxidants have been demonstrated to induce beneficial effects on human health and in disease prevention. However, the use of these antioxidants has recently been restricted due to their toxicity, low activity and various side effects.

OPC, known as condensed tannins, is widely distributed in the plant kingdom and represents a ubiquitous group of plant phenolics [2-4] and is a class of polyphenolic compounds which take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (-)-epicatechin [5,6]. Therefore, the interest in OPC, which occur widely as natural antioxidants in fruits, vegetables, nuts, seeds, flowers and bark is growing as therapeutic agents against disease involving radical damage [7-9]. Studies on the OPC of grape and grape seeds have reported a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress [3-5]. OPC from extracts of grape seeds has free radical scavenging abilities, which decrease the susceptibility of healthy cells to toxic and carcinogenic agents, inhibit low density lipoprotein oxidation as well as a variety of biological activities [3,7-11]. However, there have been no reports investigating on the OPC of wild grape seeds (WGS). It is expected that WGS would possesses antioxidant activity, similar to those reported on the study of grape seeds [2,3,7-13]. The scientific name of wild grape is *Vitis coignetiae* and is one of the grape families that usually inhabit forests in Korea, China and Japan.

Thus, in this study, the antioxidant activity of the wild grape seed extracts (WGSE) was investigated and the effective extraction conditions for their application to large scale-up processes found. Two assays; 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical bleaching [14] and the free radical scavenging activity on 1,1-diphenyl-2picrylhydrazyl (DPPH) [15], were used. The OPC contents were determined using Folin-Ciocalteu reagents [16] at each extraction step, together with their ABTS and DPPH free radical scavenging activities.

MATERIALS AND METHODS

Materials

The WGS was provided by Daejeon Health Science College (Daejeon, Korea). The OPC were extracted from the WGS (200 g) with various ratios of ethanol/water (800 mL), and at various temperatures (50~90°C) and times (1~12 h) in 2-L glass vessels with stirring by 200 rpm. Subsequently, the extract was separated by filtration

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and about 90% of the solvent removed by evaporation under reduced pressure at 50°C.

Total Polyphenol Contents

The total phenolic content of the WGSE samples were determined with the Folin-Ciocalteu reagent using (+)-catechin as a standard. The absorbance of the sample was measured at 760 nm after 30 min of reaction. The results were expressed as mg of (+)-catechin equivalents (CE) per liter.

Total Antioxidant Potential (TAP)

The total antioxidant potential (TAP) values were determined from the induction time in free radical mediated processes and the bleaching of thee stable free radical, the ABTS derived radical cation ($ABTS^{\bullet+}$). ABTS (Sigma) radical cations were prepared by incubation of 14 mM ABTS (1 mL) with 9 mM potassium persulphate (1 mL) for 12 h in 20 mM, pH 7.0, phosphate buffer. After a 10 times dilution of 3 mL of the ABTS radical cations in 20 mM phosphate buffer, 200 μL of the adequately diluted WGS extracts (WGSE) were added. The absorbance of the sample was measured at 734 nm after 30 min of reaction using 3-tertiary-butyl-4-hydroxyanisole (BHA) as the standard.

Free Radical Scavenging Activity on DPPH

The antioxidant activity of the WGSE was assessed on the basis of the scavenging activity of the stable DPPH (Sigma) free radical. 200 μL of various dilutions of the WGSE was mixed with 3 mL of ethanolic DPPH solution (5×10^{-4} M). The samples were incubated for 15 min in the dark, and the decrease in the absorbance at 517 nm was measured using a spectrophotometer using BHA as the standard. In every case, the 50% inhibition concentration (IC_{50}) values were calculated using the double integral of the signal with Eq. (1):

$$\% \text{ Inhibition ratio} = \text{ref} - \text{extract} / \text{ref} - \text{bg} \times 100 \quad (1)$$

Where ref is the reference signal (DPPH + ethanol), extract the test signal and bg the background signal. The data were the means of three repeated measurements.

Thin Layer Chromatography (TLC)

Silica-TLC (Merck) analysis of the OPC was performed in a mixture of toluene/acetone/acetic acid (3/3/1, v/v/v) [17] and visualized at 280 nm using a UV lamp with (+)-catechin used as the standard.

Column Chromatography

Chromatography was used for further purification of the OPC from the WGSE. A glass column (7.5 cm ID × 50 cm Length) was packed with Dia-ion HP-20 (packing volume 400 mL, Mitsubishi Chemical Co., Japan), and equilibrated with distilled water. After loading of the WGSE samples (75 mL, conc. 16.7 mg/mL), the column was rinsed with H_2O and 10% ethanol and the OPC eluted by 70% ethanol.

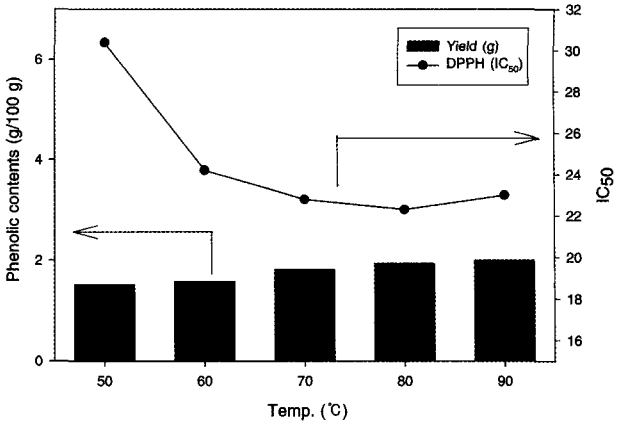

Fig. 1. The effect of temperature on the antioxidant activity and the OPC content in WGS.

RESULTS AND DISCUSSION

Effects of Temperature, Solvent Composition and Time on OPCS Extraction

OPC are known to be sensitive to extraction conditions such as temperature, solvent composition and time [18]. This work investigated the total OPC contents and total antioxidant activity of wild grape seeds and developed an efficient extraction process, with various temperatures, solvent compositions and times.

Fig. 1 shows the effect of temperature on the antioxidant activity and the OPC contents of the WGSE during solvent extraction. The total soluble phenolics in the WGSE were determined with Folin-Ciocalteu reagent according to the modified method of Singleton *et al.* [16] using (+)-catechin as a standard phenolic compound. The antioxidant activity of WGSE was determined by the DPPH free radical scavenging activity. DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [15]. The reduction capability of DPPH radicals was determined by the decrease in the absorbance at 517 nm induced by antioxidants. Hence, DPPH· is usually used as a substrate to evaluate the antioxidative activity of antioxidants. The OPC content and DPPH activity of WGSE increased with increasing extraction temperature, but decreased above 80°C as higher infusion temperatures can lead to the loss of labile polyphenolic components [19], that is, the OPC products decompose. This is consistent with the fact that the total polyphenol levels are highest when extracted at 77~80°C [19]. Fig. 2 shows the effect of solvent composition on the antioxidant activity and OPC contents in the WGS. OPC from plant materials, for analytical and preparative purposes, have been extracted

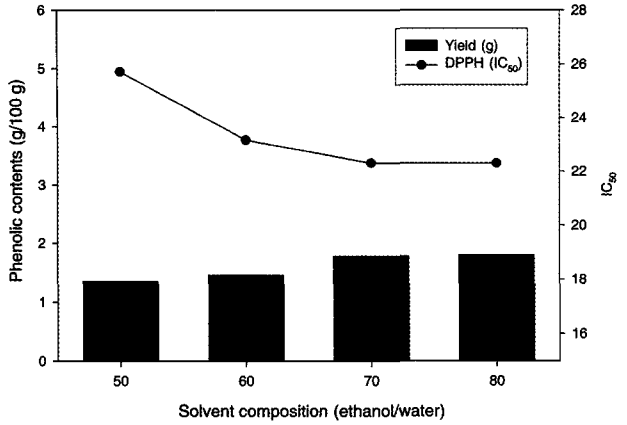

Fig. 2. The effect of solvent composition on the antioxidant activity and the OPC content in WGS.

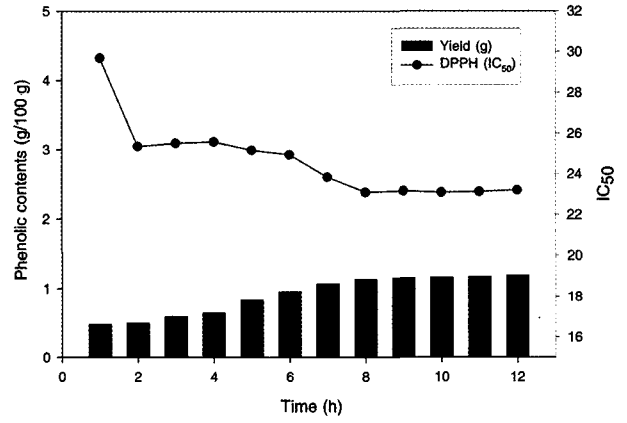

Fig. 3. The effect of infusion time on the antioxidant activity and the OPC content in WGS.

with different proportion of methanol, ethanol, and acetone, and their mixtures with water [3,16,20]. The results of the application of these solvents cannot be correlated because of the diversity of plant materials and analytical methods employed. However, from a toxicological point of view, ethanol and water are safer than acetone, methanol and other organic solvent and therefore, are more suitable within the food industry. Thus, water and ethanol extracts have been used in this study. Increases in the ethanol concentration increased the efficiency of the OPC extraction and DPPH activity. The OPC contents and activity became stable and constant above an ethanol concentration of 70%. Thus, 70% ethanol was found to be optimal for the extraction of OPC from WGS. Fig. 3 shows the effect of infusion time on the antioxidant activity and OPC contents of the WGS. The OPC content and DPPH activity of the WGSE increased with increasing extraction time, and was shown to be stable above 8 h. Therefore, the most efficient conditions for the extraction of OPC from WGS were 70% ethanol at 70°C for 8 h.

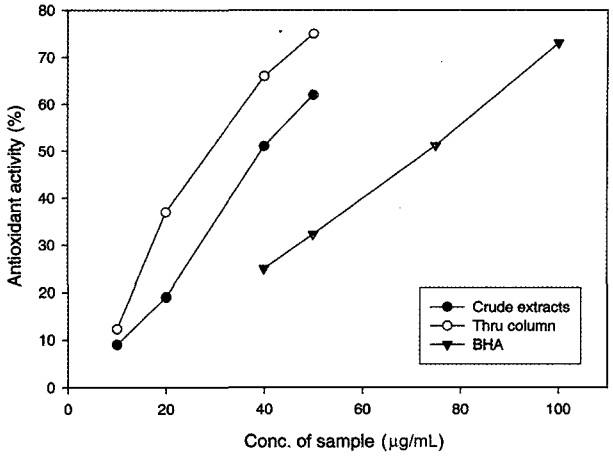

Fig. 4. The TAP of various samples at each purification step.

Purification of OPC Using Column Chromatography

The crude extracts of OPC were obtained from WGS under the optimal extraction conditions. Column chromatography was used to further purify the OPC. The WGSE was passed through a column of Dia-ion HP-20, and the column washed with H_2O and 10% ethanol. The OPC was eluted using 70% ethanol, and the residue dissolved in 95% ethanol. The total OPC contents of the final purification samples were determined using Folin-Ciocalteu reagent and expressed in g/100 g of WGS as (+)-catechin equivalents (CE), with the antioxidant activity determined by TAP and DPPH. The antioxidant activity of BHA was compared to that of standard antioxidants. Fig. 4 shows the TAP of various samples for each purification step. The TAP was determined by the bleaching of pre-formed ABTS radical cations. The addition of free radical-scavengers to a solution containing ABTS-derived radical cations leads to a decrease in the UV absorbance of the sample at 734 nm [15]. The 'thru column' sample (IC_{50}=31.60 μg/mL), the fractions of WGSE purified by column chromatography, with 70% ethanol, had higher activity than the crude extracts (IC_{50}= 40.70 μg/mL) because the impurities in the crude extracts had been effectively removed by the chromatographic procedure. The DPPH free radical scavenging activity of various samples is shown in Fig. 5. The DPPH activity of the 'thru column' sample showed the highest activity (15.70 μg/mL), whereas the IC_{50} of the 'crude extracts' and BHA were 22.90 and 32.90 μg/mL, respectively. The OPC from WGS shows significantly higher activity than the BHA with both TAP and DPPH. These results suggest that WGS has OPC that possesses higher antioxidant activity. Fig. 6 shows the TLC for the samples of crude extracts and thru column samples. Lane S is the band for (+)-catechin used as a standard. Lane T_W is the band of the 70% ethanol extracts from the WGS and Lane C_W the band of the chromatographic fractions. T_W showed various contamination spots and partially purified OPC, which had the same R_f value as catechin. C_W

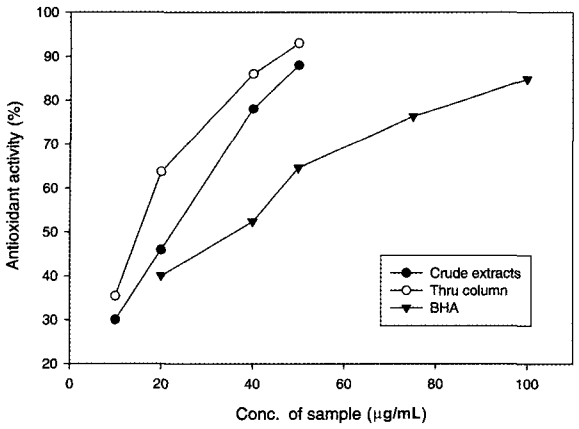

Fig. 5. The DPPH free radical scavenging activity of various samples at each purification step.

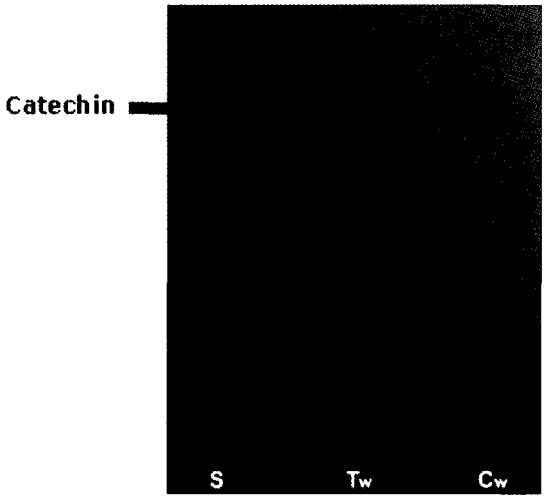

Fig. 6. The silica-TLC of OPC fractions at each purification step. S, (+)-catechin as standard, T_W, crude extracts, C_W, thru column fractions.

Table 1. Yield and activity of OPC from wild grape seeds

Purification step	[a]Yield (g)	[b]TAP (IC_{50})	[c]DPPH (IC_{50})
Crude extract	2.05	40.70	22.90
Dia-ion HP-20 Column	1.78	31.60	15.70

[a] Yield determined as catechin equivalents in g per 100 g wild grape seeds using Folin-Ciocalteu reagent; [b&c] are the inhibition concentrations (μg/mL) of DPPH and ABTS radical scavenging activities.

showed that well purified OPC were obtained because the chromatographic procedure had effectively removed the lipid, polysaccharide and contaminating proteins.

Table 1 shows the yield and activity results at each purification step. The final yield was 1.78 CE g/100 g WGS, with IC_{50} activities toward TAP and DPPH of 35.41 and 15.70 μg/mL respectively. The final activities toward TAP and DPPH were increased after the chromatographic procedure had been applied. These activities of final purified OPC were about two times higher than that of the BHA used as the reference sample. Therefore, the purification of OPC using Dia-ion HP-20 chromatography was very effective, showing higher antioxidant activities toward both TAP and DPPH.

CONCLUSION

Wild grape seeds are a rich source of OPC and have been shown to exhibit free radical scavenging activities. In this study, the effective extraction conditions to obtain OPC-rich extracts from WGS. Column chromatography was also used to further purify the OPC. The optimal conditions for the extraction of OPC from WGS were 70% ethanol at 70°C for 8 h. The OPC obtained from the column chromatography using Dia-ion HP-20 showed significantly higher activity than that of the BHA as a reference sample.

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