Effects of Water Extraction Temperatures on the Yield, Molecular Weight, and Antioxidant Activity of Proanthocyanidins Extracted from *Pinus radiata* Bark

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Abstract

Pinus radiata bark is a rich source of proanthocyanidins (PAs), a potent and valuable plant antioxidant. This study was performed to evaluate PA extraction conditions with water at various temperatures ranging from 25°C to 120°C. The properties of the water extract (WE) obtained at each temperature were investigated in terms of PA content, molecular weight (MW) distribution, and antioxidant activity. The WE yield was significantly dependent on temperature. The PA yield and the absorbance at 280 nm (an indicator of PA concentration) of WE reached maximum values at 80°C, implying increased extraction of monomeric polyphenols. Gel permeation chromatography results suggested that water extraction above 100°C caused depolymerization of extracted PAs, thereby noticeably reducing MW. It was found that more monomeric polyphenols can be extracted by increasing temperature. The WE antioxidant activity was maximized at 80°C and was dependent to some extent on the degree of polymerization.

Proanthocyanidins (PAs) are considered an important class of secondary metabolites in the plant kingdom. They are polyphenolic natural products composed of flavan-3-ol subunits linked mainly through C_4–C_8 (or C_6) bonds (Fig. 1; Kennedy and Taylor 2003, Ku and Mun 2007). Recently, PAs have received considerable attention in the fields of nutrition, health, and medicine as well as for cosmetics, largely due to their physiological activities, such as antioxidant capacity (Bros et al. 2000), antimicrobial effects (Cowan 1999), anti-inflammatory properties (Santos-Buelga and Scalbert 2000), antiallergy activity (Bagchi et al. 2000), stabilization effect on the extracellular matrix (collagen and elastin; Ku et al. 2007b, Ku and Mun 2008), and inhibitory activity against some enzymes, including phospholipase A2, cyclooxygenase, and lipoxygenase, and receptors (Zhu et al. 1997).

Pine bark is an important biomass resource, amounting to about 10 to 15 percent of the total weight of the tree (Kofujita et al. 1999). In a previous study (Ku et al. 2007a), we characterized the chemical composition of various pine barks, which are used in the wood industry and/or have been autogenously or experimentally planted in South Korea. Among the pine bark varieties used, *P. radiata* bark was considered a good source for PA extraction. We also found that temperature had a great effect on the extraction of *P. radiata* bark rather than other extraction factors such as liquor ratio and time (Jang et al. 2005). In addition, water extraction has been used industrially to produce PA-rich extracts from the bark of several types of pine. The products are currently selling in the world market under the names of Pycnogenol and Oligopin from *Pinus maritima* bark and Enzogenol from *P. radiata* bark. However, there are no detailed studies on the properties of *P. radiata* bark PAs

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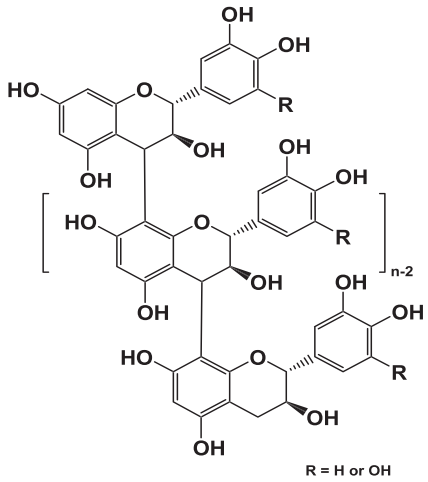

Figure 1.—Structure of proanthocyanidins in Pinus radiata *bark. R = H (procyanidin); R = OH (prodelphinidin).*

prepared using various extraction temperatures. Therefore, this study focused on the effect of temperature on water extraction of *P. radiata* bark to estimate the quality of water extracts (WEs) based on molecular weight (MW), degree of polymerization (DP), polydispersity, and antioxidant activity due to the significance of these factors in applications for pharmaceutics, cosmetics, and nutrient supplementation.

Materials and Methods

Water extractions at various temperatures

The outer bark of *P. radiata* was collected from Hanyoung Sawmill Co. in Kunsan, South Korea. The bark was dried in a convection oven at 60°C ± 1°C for 48 hours, ground using a Wiley mill, and sieved to collect a 177- to 841-μm fraction. Two grams (oven dried) of bark powder and 20 mL of deionized water were added to a 100-mL Erlenmeyer flask and placed in a water bath for 1 hour at five different temperatures (25°C, 40°C, 60°C, 80°C, and 100°C). Additionally, the water extraction was also done at 120°C in an autoclave for 1 hour. The extraction mixtures were filtered with a 1G3 glass filter and the residues were dried in a convection oven at 105°C ± 1°C for 24 hours. The WE yields were calculated from the weight losses of ovendried bark. All WEs prepared at each extraction temperature were lyophilized to dryness and kept in a refrigerator (4°C) until used.

Determination of PA content and yield

PA content in WEs was determined by vanillin-H_2SO_4 assay (Toda 2005). Aliquots (1 mL) of WEs (3 mg in 10 mL of methanol) were mixed with 2.5 mL of 1.0 percent (wt/vol) vanillin in methanol, and 2.5 mL of 25 percent (vol/vol) H_2SO_4 in methanol was added to undergo vanillin reaction. The blank was prepared by substituting 1 percent vanillin with methanol. The vanillin reaction was carried out in a water bath at 25°C for 15 minutes. The absorbance at 500 nm was read and results were expressed as (+)-catechin equivalents/WE (grams). PA yields were calculated based on bark weight by the following equation:

$$\text{PA yield}\,(\%\ \text{on bark} = (\text{PA content} \times \text{WE yield})/100 \quad (1)$$

MW distribution

WE was acetylated by dissolving with 1 mL of pyridine–acetic anhydride (1:1, vol/vol) for 48 hours at ambient condition. One milligram of the acetylated WE was dissolved in 1 mL of tetrahydrofuran and subjected to gel permeation chromatography (GPC) analysis. MW was estimated using a Spectra-Physics modular LC system equipped with an SP 8800 ternary HPLC pump, a Spectra 100 variable wavelength detector, and a combination of Shodex GPC KB-802.5 (8 by 300-mm) and AM GEL GPC (10 by 300-mm) columns purchased from American Polymer Standards. The mobile phase was tetrahydrofuran at a flow rate of 1.0 mL/min. The effluent from the column was continuously monitored at 280 nm for the sample and 254 nm for the standard. MW calculations were based on a calibration curve obtained using monodisperse polystyrene standards, acetylated (+)-catechin, and phenol. MW of the substituted acetyl group was excluded in this calculation based on procyanidin units. A calibration curve was constructed by second-order, polynomial regression analysis with coefficients C_0, C_1, and C_2. The coefficient of variation was 0.998. Values for retention time (t_r) and the corresponding absorbance (A) values were imported into a worksheet in Excel. M_r, molecular weight corresponding to each t_r value, was calculated from the calibration curve using Equation 2.

$$\log(M_r) = C_0 + C_1(t_r) + C_2(t_r)^2 \quad (2)$$

The M_r values obtained were transformed into number-average molecular weight ($\bar{M}n$), weight-average molecular weight ($\bar{M}w$), and polydispersity (PD) values using the absorbance values as given by Equations 3, 4, and 5.

$$\bar{M}n = \sum A_i M_i \Big/ \sum A_i \quad (3)$$

$$\bar{M}w = \sum A_i M_i^2 \Big/ \sum A_i M_i \quad (4)$$

$$PD = \bar{M}w/\bar{M}n \quad (5)$$

The DP of PAs was determined by dividing $\bar{M}w$ or $\bar{M}n$ into procyanidin units (MW = 288), where we assumed that the PA value was completely composed of procyanidin units linked mainly through C_4–C_8 bonds.

Antioxidant activity

WE was diluted to 17 μg/mL concentration in methanol. One milliliter of the diluted solution was mixed with 2 mL of 0.1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl radical) and reacted at 25°C for 30 minutes. Afterward, the absorbance of the solutions was read at 518 nm. The blank was methanol and the control was a mixed solution of 1 mL of methanol and 2 mL of 0.1 mM DPPH. Antioxidant activity was expressed as the scavenged amount of DPPH free radical. The mean and standard deviation (SD) of these results were calculated.

$$\text{DPPH free radical scavenging activity}\,(\%) = [1 - (\text{absorbance of sample}/\text{absorbance of control})] \times 100 \quad (6)$$

Results and Discussion

Figure 2a shows the WE yield from *P. radiata* bark as a function of temperature. The water extraction plot was sigmoidal and confirmed the temperature dependence of extraction. Increasing temperature may favor extraction by enhancing the solubility of phenolic compounds in water and may also increase the rate of extraction and hence decrease the extraction time. The extraction of the pine bark using hot compressed water at 120°C gave a very similar extract yield to that of the conventional hot water extraction at 100°C under ambient pressure. Temperature influences the oxidation of polyphenols, which proceeds slowly at room temperature but rapidly under conditions of high temperature and high humidity (Janick and Simon 1993). This supports the assumption that hot water extraction may cause a comparatively rapid oxidative reaction, producing a secondary substance as a type of insoluble polymeric polyphenol. On the other hand, the polyphenol content in *P. radiata* bark accounts for 55 percent of the bark (Ku et al. 2007a). Therefore, it was thought that the polyphenols such as nonextractable PAs that are found in pine barks, particularly in the outer bark (Matthews et al. 1997), are not extracted by the hot compressed water because the WE and the PA yields are still very low even at high temperature extraction. Figure 2b shows the PA content in WE obtained at each temperature. The PA content decreased about 15 percent when the temperature increased from 25°C to 120°C. This result indicates that other components in the bark were extracted more with increasing temperature, since the PA content was reduced despite the increased WE yield. Figure 2c shows the WE absorbance at 280 nm as a function of temperature. The absorbance at 280 nm, an indicator of PA concentration, increased with increasing extraction temperatures, and reached a plateau at 80°C, indicating that the extraction of other components increased at temperatures between 80°C and 120°C. Water extraction at a lower temperature resulted in WEs with a high PA content, but the PA yield may have been lower due to the low WE yield. Figure 2d shows PA yield at various extraction temperatures. The PA extracted from the bark increased with increasing temperatures and reached a maximum at 80°C. A similar finding has also been reported for the extraction of oligomeric PAs from wild grape seeds (Huh et al. 2004). These results suggested that extraction of PAs increases with increasing temperature, but at temperatures above 80°C, other components are extracted more than PAs. The results confirmed that extraction temperature is an important factor for water-based extraction of PAs from *P. radiata* bark, where PAs becomes partially insoluble and nonreactive components become soluble (Inoue et al. 1998).

Figures 3 and 4 show the effects of extraction temperature on the WE MW distribution and on MW, DP, and polydispersity ($\bar{M}w/\bar{M}n$). The MW of WE increased up to 80°C and then decreased at 100°C and 120°C (Fig. 4). Such changes were probably caused by a depolymerization of higher MW PAs extracted out of the bark into the solution during the batch extraction. An increase in the monomeric polyphenols (20.5 min) with absorbance at 280 nm was evident with increasing temperature (Fig. 3). This result and that of a previous related study (Mun and Ku 2006) confirmed that the other components extracted at temperatures above 80°C are monomeric polyphenols containing phenolic acids and flavonoids. The depolymerization above 100°C may have the advantage of producing a preliminary removal of high MW PAs with the potentially strong reactivity, thereby preventing WE quality deterioration. The remarkable reduction in DP and MW of WE was observed

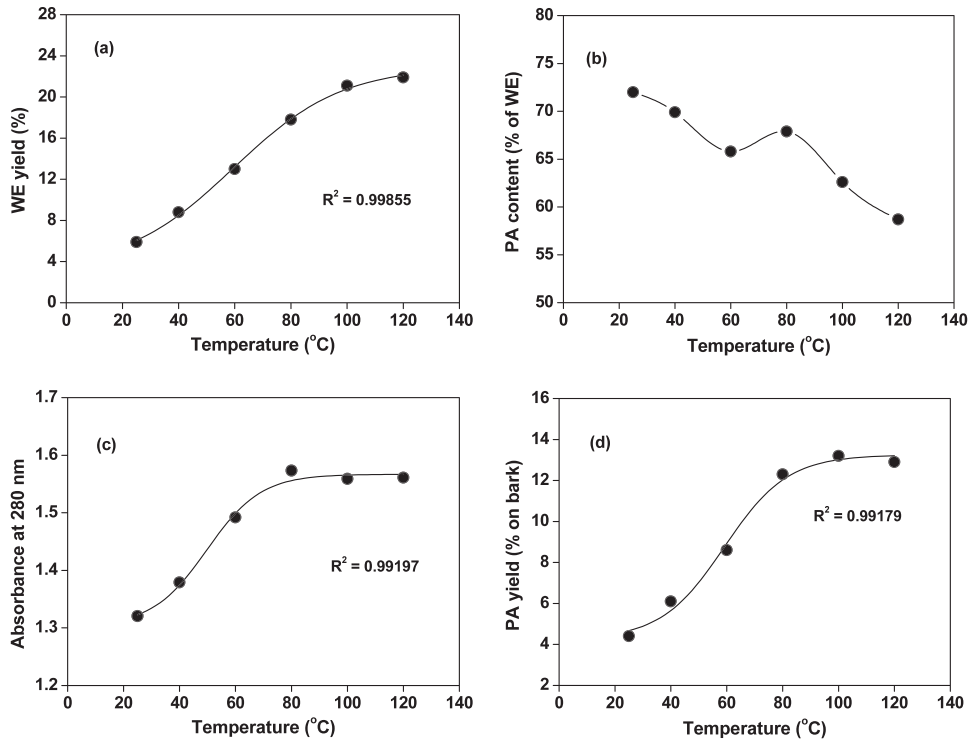

Figure 2.—Temperature effect on water extract (WE) yield (a), proanthocyanidin (PA) content (b), water extract absorbance at 280 nm (c), and PA yield (d) from Pinus radiata *bark.*

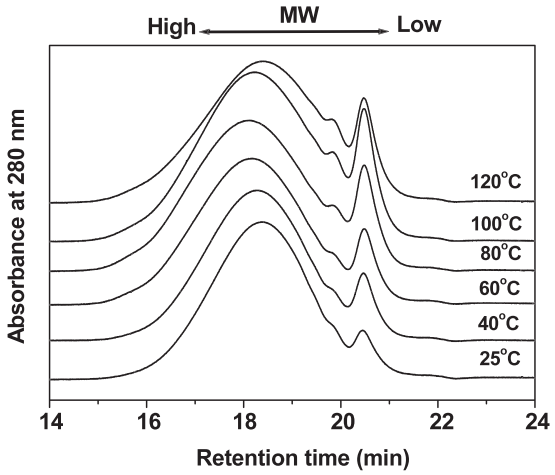

Figure 3.—Molecular weight (MW) distribution of water extracts from Pinus radiata *bark at increasing temperatures.*

above 100°C (Table 1; Fig. 4). These results may also support the commercial application as a cosmetic material or pharmaceutical drug to some extent because increased polymerization is characterized by poor absorption through the skin or gut barrier (Prior and Gu 2005).

Figure 5 shows the antioxidant activity value estimated from the DPPH free radical scavenging activity of WE. The antioxidant activity increased with increased extraction temperature; it reached a maximum at 80°C and then decreased. The antioxidant activity of WE is mainly due to PAs because they are major components in WE. Although some monomeric polyphenols in WE also affect the activity, the effect would be small because PAs are a more potent antioxidant than the monomeric polyphenols (Yokozawa et al. 1998). The antioxidant activities of an extraction temperature above 80°C caused lower DPPH radical scavenging activity in spite of the similar PA yield, as shown in Figure 2d. This could be explained by the secondary extraction of other components from bark at a higher temperature (Fig. 2a). From this result, it was thought that the lower antioxidant activities at 100°C and 120°C are mainly due to the increased secondarily extracted components, which have little or no DPPH scavenging ability. A secondary oxidative reaction above 100°C also may reduce the antioxidant activity because of a structural transformation from unoxidized B-type PAs to oxidized A-type PAs as

Table 1.—Yields of water extract (WE), molecular weight (MW), polydispersity, and degree of polymerization (DP) of WEs from Pinus radiata *bark.*[a]

Extraction temperature (°C)	WE (%)	$\bar{M}w$	$\bar{M}n$	Polydispersity ($\bar{M}w/\bar{M}n$)	DP	
					$\bar{M}w$	$\bar{M}n$
25	5.9	2,930	890	3.3	10.2	3.1
40	8.8	3,050	900	3.4	10.6	3.1
60	13.0	3,610	880	4.1	12.5	3.1
80	17.8	3,690	820	4.5	12.8	2.8
100	21.1	3,290	760	4.3	11.4	2.6
120	21.9	3,210	730	4.4	11.1	2.5

[a] Extraction conditions of WE: temperature, 25°C to 120°C; time, 1 hour; water-to-bark ratio, 10:1.

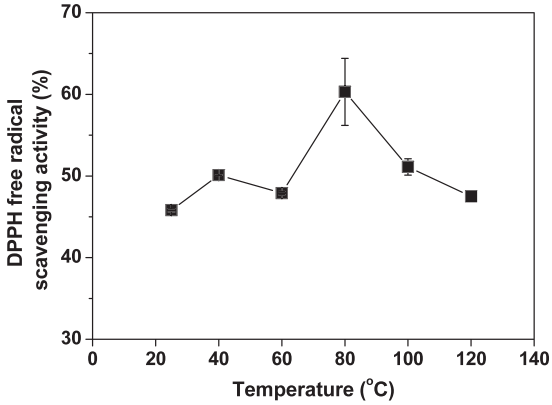

Figure 5.—Temperature effect on antioxidant activity of water extracts from Pinus radiata *bark.*

described by Kondo et al. (2000). Kondo et al. (2000) reported that the antioxidant activity of B-type PAs is greater than that of A-type PAs. Meanwhile the antioxidant activity was also found to be dependent on DP of PAs at extraction temperatures below 80°C. It was reported that increasing DP may enhance the effectiveness of PAs against DPPH free radicals (De Bruyne et al. 1999, Heim et al. 2002).

Conclusions

The water extraction of *P. radiata* bark was performed in the temperature range of 25°C to 120°C, and the following conclusions were reached from this study:

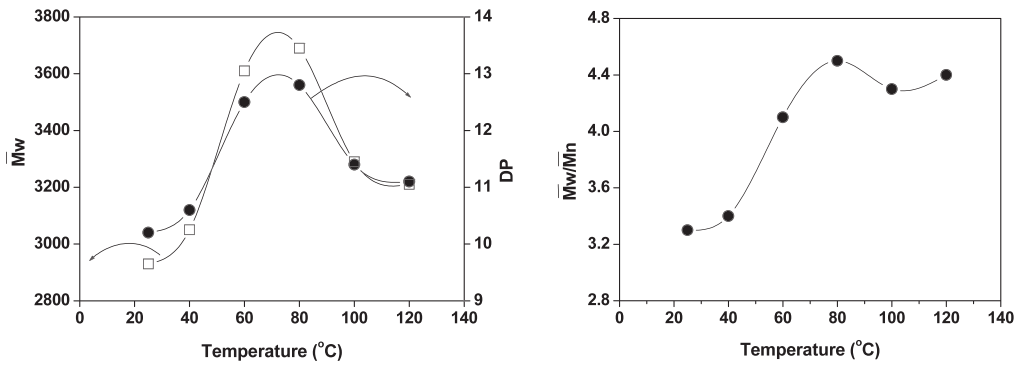

Figure 4.—Temperature effect on molecular weight (MW), degree of polymerization (DP), and polydispersity ($\bar{M}w/\bar{M}n$) of proanthocyanidin-rich water extracts from Pinus radiata *bark.*

1. The water extraction temperature was a significant factor affecting the extraction of PAs and their related components. The sigmoidal plot was appropriate for relating the temperature-dependent extraction ($R^2 \fallingdotseq 0.99$).
2. The temperature was an important factor for the MW and DP of PAs extracted in the range of 80°C to 100°C.
3. The antioxidant activity of WE peaked at 80°C and showed a somewhat DP-dependent tendency.
4. The temperature of 80°C to 100°C was determined to be the desirable range for PA extraction, but water extraction at 100°C showed greater practical potential because the cosmetic material and pharmaceutical drug industries need PAs with a lower DP due to the importance of penetration.

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