Effects of Severity Factor on the Subcritical Water Treatment of Polyphenols Obtained from Japanese Persimmon

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Subcritical water treatment at temperatures of 140–220\textdegree C was applied to polyphenols obtained from Japanese persimmon. Properties of the treated polyphenol solution, such as soluble and insoluble solid contents, freezing-point depression, and DPPH radical scavenging activity, were measured. All tested properties were well expressed by the severity factor reflecting the quantity of energy loaded onto the persimmon polyphenols. At a greater severity factor, precipitation occurred and the soluble solid content decreased. However, polyphenol depolymerization occurred simultaneously owing to the formation of acidic compounds, resulting in greater freezing-point depression. The antioxidative activity of the treated solution was also evaluated by measuring the DPPH radical scavenging activity, and was found to increase with an increase in the severity factor.

Keywords: antioxidative activity, depolymerization, persimmon polyphenol, polymerization, subcritical water treatment

Introduction

Persimmon (\textit{Diospyros kaki} Thunb.), belonging to the Ebenaceae family, has been cultivated mainly in China, Korea, and Japan, with an annual production of ca. 4.5 million tons in 2012 (Kometani \textit{et al.}, 2016). Persimmon contains many nutrients such as vitamin C, carotenoids, and dietary fibers (Inakuma, 2016). There are two types of persimmons, astringent and non-astringent, based on when the loss of astringency occurs naturally in the fruit. Persimmons are often eaten as is, or in the case of astringent persimmon, after deastringency has been achieved. Unlike other fruits, the use of persimmons for food processing, such as juice production or baking, has been limited. Therefore, further uses of persimmon should be focused upon.

There are many kinds of polyphenols, several of which have been reported to show various bioactivities, such as antioxidative activity (Koga \textit{et al.}, 1999), antihypertensive activity (Grassi \textit{et al.}, 2005), hyperglycemia inhibition activity (Grassi \textit{et al.}, 2005; Yamashita \textit{et al.}, 2012; Schulze \textit{et al.}, 2014), and anticancer activity (Bagchi \textit{et al.}, 2014). Immature and mature astringent persimmon fruits contain persimmon polyphenols (proanthocyanidins), a kind of polymerized polyphenols, in a low molecular mass, leading to a peculiar astringency. However, persimmon polyphenols exist as macromolecules in the mature fruit of sweet persimmon, and in the deastringent fruit of astringent persimmon (dried persimmon) (Taira \textit{et al.}, 1997). Macromolecular persimmon polyphenols have low solubility in water, resulting in the lower astringency. This makes it convenient to eat the persimmon as it is; however, the low solubility would limit bioavailability of persimmon polyphenols to human body. To overcome this drawback, depolymerization of the persimmon...
polyphenols could be a promising approach.

Subcritical water is water kept in its liquid state at temperatures of 100–374°C under high pressure conditions, and has two distinct properties, low dielectric constant and high ion product (Yoshida et al., 1999; Tavakoli and Yoshida, 2006; Pourali et al., 2009). These properties contribute to the efficient extraction of hydrophobic substances from biomass and to the high reactivity towards many compounds (Wiboonsirikul et al., 2007; Pourali et al., 2009; Khuwijitjaru et al., 2014; Kambara et al., 2016). It has been reported that polyphenol decomposition occurred during subcritical water treatment (Pourali et al., 2010), implying that the same may occur during the depolymerization of persimmon polyphenols.

In this study, we performed the subcritical water treatment of persimmon polyphenols to produce polyphenol oligomers with lower molecular mass. The role of subcritical water in polyphenol depolymerization was investigated, and sample properties, such as antioxidative activity, were also evaluated.

Materials and Methods

Materials Preparation of polyphenols from persimmon fruits was carried out as described previously (Hamasaki, 2010). Briefly, immature fruits of ‘Tone-wase’, a kind of persimmon cultivated in Japan, were harvested, then treated with ethanol to remove astringency, and homogenized with a juice extractor. These homogenates were centrifuged at 1,630 x g for 15 min and showed 4 layers in the centrifuge tubes. The second layer from the bottom, which contained so-called tannin cells, was separated, and lyophilized. The dried powders were suspended in water at ambient temperatures in order to remove lower molecular mass constituents, and then the resultant precipitate was extracted with water at 121°C for 15 min. Solubilized polyphenols were lyophilized again, and used as starting material in this study. Persimmon polyphenols were shown to comprise 70% polyphenols, 20% sugars, and 10% unknown components.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were also obtained from Wako.

Subcritical water treatment of persimmon polyphenols Persimmon polyphenols were dissolved in distilled water at a final concentration of 5% (w/v). The solution was centrifuged at 7,100 x g for 10 min at 4°C. The supernatant was filtered through a No. 1 filter paper (Advantec Toyo, Tokyo, Japan) and subsequently through a membrane filter (DISMIC-25CS, 0.8 μm, Advantec Toyo) to obtain a polyphenol solution.

Subcritical water treatment of the polyphenol solution was carried out in a tubular reactor similar to that conducted in a previous study (Kambara et al., 2016). The reactor consisted of a SUS 316 HPLC tube (0.8 mm I.D. x 4 m) connected to an LC-10AT HPLC pump (Shimadzu, Kyoto Japan) and a back-pressure regulator (P-880, Upchurch Scientific, Oak Harbor, WA, USA). The inlet-side part of the tube (2 m in length) was immersed in a silicone-oil bath (140–220°C) to begin subcritical water treatment, and another part (0.6 m in length) was dipped in a water bath to terminate the reaction. The sample temperature was assumed to reach the temperature of silicone-oil or water immediately because of large thermal conductivity and small diameter of stainless steel tube. The polyphenol solution was delivered into the reactor for the prescribed residence time (0.5–10 min) to obtain the treated solution. The untreated solution was used as the control.

Measurement of soluble and insoluble solid content The soluble and insoluble solid contents of the treated and control solutions were analyzed. The solutions were centrifuged at 6,100 x g for 3 min. The supernatant was withdrawn for analysis of the soluble matter, and the residual solvent in the precipitate was removed under reduced pressure to obtain the dried insoluble matter. Soluble matter content was measured by the oven drying method. One milliliter of the supernatant was placed in a 5-mL vial, which was heated at 105°C for 2 h in an oven to remove residual water. Soluble and insoluble solid contents were determined on the basis of the weight of the dried residue and were expressed as mg-soluble solid/g-solution or mg-insoluble solid/g-solution.

Molecular mass distribution To estimate the degree of depolymerization of persimmon polyphenols, molecular mass distribution was measured by gel permeation chromatography (GPC). The control and treated solutions (1 mL) were centrifuged at 6,100 x g for 2 min, and the resulting supernatant was used for GPC analysis. GPC analysis was performed using a diol column (YMC-pack Diol-60, 8.0 mm I.D. x 500 mm, YMC, Kyoto, Japan) equipped with an LC-20AD HPLC pump (Shimadzu) and an RID-10A refractive index detector (Shimadzu). The eluent was distilled water with a flow rate of 1.0 mL/min. A calibration curve between the molecular mass and elution volume for the Diol-60 column from the catalog (YMC, 2007) was used for reference.

Freezing-point depression of the control and treated solutions was measured using an osmometer (OM802, Vogel, Kevelaer, Germany) to evaluate the degree of persimmon polyphenol depolymerization.

Measurement of UV absorbance The control and treated solutions (1 mL) were centrifuged at 6,100 x g for 2 min. The resulting supernatant was diluted 50–1000 times with distilled water to prepare the sample solutions. The absorbance of the solutions was measured at 453 nm using a U-5100 spectrometer (Hitachi High-Tech Science, Tokyo, Japan).

Antioxidative activity The control and treated solutions (1 mL) were centrifuged at 6,100 x g for 2 min. The resulting supernatant was diluted 100 times with distilled water to prepare the sample solutions. Trolox was dissolved in 95% (v/v) aqueous ethanol at concentrations between 5–100 μmol/L. The samples, Trolox solutions, and 95% (v/v) aqueous ethanol as a blank (0.75 mL each) were added in separate 2.0 mL-black tubes. To each of these
solutions 0.75-mL of 0.15 mmol/L DPPH in 95% (v/v) aqueous ethanol was added. The sample was left in the dark at room temperature for 30 min. Absorbance of the sample at 517 nm was measured using a Hitachi U-5100 spectrometer. Radical scavenging activity of the solution was defined by the following equation:

Radical scavenging activity (%) = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100 \quad \text{Eq. 1}

where \( A_{\text{blank}} \) and \( A_{\text{sample}} \) are the absorbance of blank and sample solutions, respectively. Radical scavenging activity was then calculated based on Trolox equivalent (μmol/g-soluble solid).

Results and Discussion

Soluble and insoluble solid contents In subcritical water treatment, temperature and time are generally considered the operating variables. However, the thermal history of the treatment greatly influences properties of the treated solution. The severity factor, \( R_0 \), has been suggested as a parameter reflecting the thermal history, (Heitz et al., 1987), and is defined by the following equation:

\[ R_0 = \int_0^t \exp \left( \frac{T(t) - 100}{14.75} \right) dt \quad \text{Eq. 2} \]

where \( T \) is the treatment temperature (°C) at time \( t \) (min), the value of 100 is the base temperature (°C), and the value 14.75 (min) is an empirical parameter related to the activation energy assuming a first-order reaction.

In this study, properties of the treated solution were assumed to depend on the quantity of energy loaded onto persimmon polyphenols, and we tried to correlate these properties with the severity factor. Figure 1 shows the effect of the severity factor on soluble and insoluble solids contents in the solution obtained by the treatment of persimmon polyphenols. Regardless of treatment temperature and time, a curve was obtained for each plot for the property vs. severity factor. Significant precipitation was observed on treatment at higher temperatures. Furthermore, insoluble solid content abruptly rose with an increase in the severity factor, \( R_0 > 500 \) min, corresponding to a treatment temperature range of 160–220 °C. The insoluble solid content reached 22.5 mg/g-solution at \( R_0 = 1.7 \times 10^4 \) min. Precipitation could be ascribed to the polymerization of phenolic compounds in addition to caramelization of sugars during treatment. Conversely, when the severity factor exceeded 500 min, the soluble solid content decreased with an increase in the severity factor, possibly owing to precipitate formation. The decrease in soluble solid content corresponded well with the increase in insoluble solid content, suggesting that gasification, such as decarboxylation, did not occur during the treatment.

Change in molecular mass during subcritical water treatment

Measurement and GPC analysis of the freezing-point depression of the treated solution were performed to evaluate a change in the molecular mass of persimmon polyphenols during subcritical water treatment. Figure 2 shows GPC chromatograms of the solutions obtained by treatment at 180 °C (\( R_0 = 113–1134 \) min). The chromatogram for the control solution contained a single peak (\( M_w \approx 10^5 \)). When the analysis was performed for the treated solution at 180 °C, some broad peaks were noted with smaller molecular masses around a peak top of \( M_w \approx 4 \times 10^4 \). Depolymerization was observed to proceed significantly with an increase in the severity factor.

Figure 3 shows the effects of severity factor on the freezing-point depression of the treated solutions. The plot of freezing point depression against severity factor could be expressed as a curve with any of the treatment conditions. The freezing-point depression did not change when the treatment was performed at 140 °C. However, the solutions treated at 160–220 °C (\( R_0 > 100 \) min) exhibited a greater depression with an increase in the severity factor, especially when it was more than 800 min, corresponding to
the treatment temperatures of 200–220°C. These results suggest the formation of small molecules, and that depolymerization could be promoted with an increasing amount of heat applied on persimmon polyphenols. It was reported that subcritical water extraction of defatted rice bran gave higher total phenolic contents of the extract at higher treatment temperatures (≥ 200°C) (Wiboonsirikul et al., 2007). Decomposition of lignin could result in higher total phenolic content in this case. These facts suggest that temperatures of at least 160–180°C would be required to sufficiently decompose polymerized polyphenols into low molecules, and that decomposition at a reduced rate occurred at temperatures lower than 160°C. In addition, these results also indicate that persimmon polyphenols and sugars disproportionated to yield the decomposed products with lower molecular mass and the insoluble matter (humin). It was reported that humin usually formed as the result of polymerization occurring during the subcritical water treatment (Asghari and Yoshida, 2006).

The effect of severity factor on the pH value of the treated solution was evaluated (Fig. 4). The pH values decreased from 4.4 to 3.9 with increase in severity factor from 10 to 1000 min, and when \( R_0 > 1000 \) min, it leveled off. This could be ascribed to the decomposition and oxidation of the components resulting in the formation of acidic compounds, such as formic and acetic acids (Luck, 1999; Asghari and Yoshida, 2006).

It was reported that the C-C bond constituting the polymerized polyphenols was cleaved in the presence of an acid catalyst (Pourali et al., 2010). Freezing-point depression remarkably increased when the severity factor was within the range of 100–1000 min as shown in Fig. 3. This could be a result of the decomposition of persimmon polyphenols with the formation of organic acids. Therefore, the freezing-point depression was plotted against the pH value of the treated solutions to clarify the role of these acids (Fig. 5). Freezing-point depression gradually became greater with decreasing pH values from 4.5 to 3.9, and it abruptly rose when the pH value reached 3.9 forming an inflection point on the plot. Severity factor corresponding to this point was ca. 1500 min, and this point denotes the condition at which decomposition of the polyphenol to a low molecule begins to proceed significantly. This would also mean that formation of the organic acids greatly contributed to the formation of low molecules.

Relationship between the color of the treated solution and severity factor  The effects of severity factor on the coloration of the treated solution was evaluated by measuring the absorbance of the treated solutions at 453 nm (Fig. 6). When severity factor was less than 3000 min, the absorbance increased, whereas browning became significant with an increase in the severity factor. This effect could be due to the polymerization and caramelization of the constituents. The absorbance then decreased when \( R_0 > 3000 \) min, reflecting a decrease in content of the soluble matter in the treated
solution due to the progressing precipitation.

Effects of subcritical water treatment on the antioxidative activity  Antioxidative activity of the treated solution was evaluated by the change of DPPH radical scavenging activity during the treatment. Figure 7 shows the relationship between the severity factor and radical scavenging activity. The radical scavenging activity decreased when the severity factor was less than 1700 min. At greater severity factor ($R_0 > 1700$ min), radical scavenging activity increased with an increase in the severity factor. It became more than 200 μmol/(g-soluble solid) when $R_0$ was more than $5.0 \times 10^3$ min. Although depolymerization began even at 100 min of severity factor based on the freezing-point depression (Fig. 3), increase of the radical scavenging activity became remarkable when the severity factor was more than 1700 min, suggesting that radical scavenging activity of the polyphenols improved only when depolymerization had sufficiently progressed. In addition, byproducts formed by caramelization, such as 5-hydroxymethyl furfural, would also exhibit radical scavenging activity (Luo et al., 2009).

In conclusion, subcritical water treatment of persimmon polyphenols could promote depolymerization and improve the antioxidative activity of the treated solution. Although the treatment temperature and time affected all the measured properties, they could be simply summarized by the severity factor, suggesting its effectiveness in the subcritical water treatment.

References


