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ARTICLE

Phenolics Enrichment Process from Unripe Apples

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Abstract Unripe apples contain significant amount of phenolics with various health benefits. A pilot scale enrichment process of unripe apple phenolics with Viscozyme L extraction and XAD-7 sorption process, based on the total phenolic content (TPC), phenolics content by high-performance liquid chromatography analyses, and antioxidant activities were studied. Antioxidant activities were tested by measuring oxygen radical absorbing capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, and ferric-reducing antioxidant power (FRAP). The phenolics-rich final product, apple antioxidant phenolics (AAP) has 771-fold higher TPC and 600-fold higher antioxidant activity of unripe apples. Through XAD-7 sorption AAP showed 52, 87, 70 and 44-fold increases in TPC, ORAC, DPPH and FRAP values, respectively. AAP showed more than 95% stability at the temperature range of 20 to 120°… and pH range of 1.47 to 8.5. Apple phenolics extraction process of Viscozyme L extraction coupled with XAD-7 sorption could be applied to the production of health benefit antioxidants.

Keywords antioxidant · phenolics · sorption · unripe apples · Viscozyme L

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Introduction

Unripe apples, which refer to fallen and thinned-out apples in orchards, have much higher contents of antioxidant phenolics than ripe apples (Marian et al., 2000; Park et al., 2004; Akiyama et al., 2005; Renard et al., 2007). Based on the profile of the phenolics composition of Fuji apples during the period of apple fruit growth, the most efficient picking date of unripe apples were suggested as 85th day after full bloom day (DAFB) (Zheng et al., 2012). Apple phenolics are constituted with various classes of compounds, *e.g.* flavan-3-ols (catechin, epicatechin, and proanthocyanidin), hydroxycinnamic acids (chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid), dihydrochalcones (phloridzin and phloretin), and flavonols (quercetin and rutin) (Mohamed et al., 2001; Schieber et al., 2003; Alonso-Salces et al., 2005). The phenolics extraction yield of unripe apple increased about 2-folds with optimized Viscozyme L treatment as the ratio of Viscozyme L to substrate 0.0195 (1.95 FBG), reaction temperature 47.12°C, and reaction time 12.52 h (Zheng et al., 2010). However, the hydrolyzed solution from unripe apple contains high ionic and polar components including carbohydrates and organic acids. Recently, the isolation and enrichment of plant phenolics by the adsorption-desorption process using highly efficient resins, such as styrene-divinylbenzene copolymers are introduced to remove unwanted polar compounds (Zhao et al., 2008; Miriam et al., 2009). Hence, we studied a pilot scale of the enrichment process of unripe apple phenolics using Viscozyme L extraction and adsorption processes on the basis of total phenolic content (TPC) and antioxidant activity.

Materials and Methods

Plant sample materials. Unripe apples (*Malus pumila* cv. Fuji) were collected from the orchard of Kyungpook National University, Korea, in July 6, 2010 (85 DAFB), and stored in a freezer (70°C) until the experiment.

Reagents and instruments. Viscozyme L (from *Aspergillu saculeatus*, 100 fungal beta-glucanase units (FBG)/mL) was

purchased from Novozymes Co. (Denmark). Folin-Ciocalteau phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), chlorogenic acid, caffeic acid, *p*-coumaric acid, quercetin-3-glucoside, phloretin, and phloridzin were obtained from Sigma Co. (USA). Amberlite XAD-7 resin was purchased from Rohm & Haas Co. (France). All organic solvents were purchased from Merck Co. (Germany), except solvents for high-performance liquid chromatography (HPLC) were purchased from J.T. Baker Co. (USA). Other chemicals were purchased from Duksan Co. (Korea). A UV-Visible spectrophotometer (UV 1601 PC, Shimadzu, Co., Japan), an HPLC (LC-10A, Shimadzu, Co.,) associated with the UV-Visible detector (SPD-10A, Shimadzu, Co.,), and a Victor3 1420 multilabel counter (PerkinElmer Inc., USA) were used for the unripe apple phenolics analyses and antioxidant assays.

Enzyme-aid extraction from unripe apples. Ten kilograms of whole unripe apples were blanched at 85°C for 15 min for the inhibition of polyphenol oxidase (PPO) (Buckow et al., 2009), crushed, and homogenized with equal volume of water. After addition of 1.95% Viscozyme L (1.95 FBG), the homogenized solution (unripe apple crude solution, ACS) was incubated at 47.1°C, for 12.5 h (Zheng et al., 2010), filtered, and concentrated to apple crude phenolics (ACP) with a rotary evaporator (EYELA N-21S, Tokyo Rikakikai Co., Ltd., Japan). The extraction processes were carried out in triplicate.

Enrichment of unripe apple phenolics. ACP was applied onto Amberlite XAD-7 column (100×7 cm), washed out with deionized water, and eluted with 95% ethanol. The eluted solution was collected, concentrated, and freeze-dried at 2.5×10^{-6} Torr for 60 h (PVT FD 20R, Ilshin Co, Korea). The schematic diagram of the phenolics extract and enrichment process from unripe apples is shown in Fig. 1. The eluted solutions were analyzed by HPLC for the adsorption and desorption efficiencies, as well as yields. The yields were defined as the ratios between the measured and initial quantity of the phenolics. All enrichment processes were carried out in triplicate.

TPC determination. Total phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999), and expressed as gallic acid equivalents (mg GAE/kg).

HPLC analysis. Twenty microliters of each test solution was injected onto HPLC equipped with an ODS-HG-5 (Develosil, 150 ×4.6 mm i.d.) column, in a mobile phase of 2% (v/v) acetic acid in water (solvent A) and 0.5% (v/v) acetic acid and 49.5% (v/v) acetonitrile in water (solvent B) at a flow rate of 1.0 mL/min, and monitored at 290 nm.

Antioxidant activity determination. Oxygen radical absorbance capacity (ORAC), DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP) were examined by the methods of Zheng et al. (2012). The values were expressed as mmol Trolox equivalents per gram of each sample (mmol TE/g).

Thermal and pH stability examination. Thermal and pH stabilities of the phenolics-rich products from unripe apples were examined as the residual ratios of caffeic acid content (CAC) by HPLC determination. For the examination, the solutions were kept in an oven at temperature range of 25 to 140°C, and maintained at pH range of 1.47 to 8.5 using acetic acid or NaOH solution for 1 h, respectively (Seok and Kim, 2003).

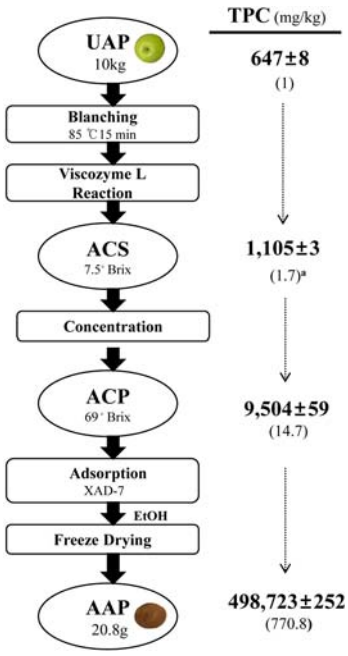

Fig. 1 Schematic diagram of the phenolics enrichment process from unripe apple[a]. [a]UAP: unripe apple, ACS: unripe apple crude solution, ACP: apple crude phenolics, AAP: apple antioxidant phenolics, TPC: total phenolic content.

Statistical analysis. All tests were carried out in triplicate, and the values were presented as mean ± standard deviation (SD). Statistical analyses were performed by Statistical Analysis System (SAS version 9.1, SAS Institute Inc.USA, 2003). The values were evaluated by the analysis of variance, followed by Duncan's multiple range tests (p <0.05).

Results and Discussion

Pilot scale enrichment process of unripe apple phenolics. Because apple phenolics are mainly contained in the peel and seeds, most of the phenolics are left in the pomace during juice production (Zheng et al., 2008). Therefore, the enzyme-aided extraction of unripe apple phenolics using Viscozyme L was attempted (Zheng et al., 2009) and optimized (Zheng et al., 2010). After blanching for the inhibition of PPO, unripe apples were hydrolyzed at the optimized conditions for 12.52 h and ACS was obtained. The diverse composition and high content of phenolics with Viscozyme L treatment were observed and reported (Zheng et al., 2009). In addition to Viscozyme L treatment, XAD-7 adsorption was introduced to the process, and the increment profile of the phenolics contents in each extraction step are shown in Table 1. Although TPC in ACS increased by 1.7-fold, the

Table 1 Phenolics content of unripe apples and their processed products (mg/kg)

Product[1)]	Total phenolic content	Chlorogenic acid	Caffeic acid	*p*-coumaric acid	Phloridzin	Phloretin
UAP	646.91±8.0[a2)]	129.07±3.46[a]	3.21±0.05[a]	1.83±0.01[a]	54.89±2.31[a]	0.98±0.35[a]
ACS	1105.24±3.07[b]	8.70±0.34[b]	43.13±0.45[b]	20.70±0.24[b]	27.41±0.11[b]	25.28±0.13[b]
ACP	9504.57±58.74[c]	96.04±3.21[c]	410.69±2.34[c]	112.83±1.97[c]	115.34±4.23[c]	20.22±1.34[c]
AAP	498723.21±252.42[d]	7297.2±53.78[d]	36156.7±36.68[d]	10223.8±82.32[d]	8725.6±45.97[d]	3159.4±54.65[d]

[1)]UAP: unripe apple, ACS: unripe apple crude solution, ACP: unripe apple crude phenolics, AAP: unripe apple antioxidant phenolics.
[2)]The results are expressed as the mean ± SD. (n =3). Values followed by different lowercase letters within the same column are significantly different at p <0.05.

caffeic acid, *p*-coumaric acid, and phloretin contents increased up to 13-, 11-, and 25-fold compared to unripe apple (UAP), respectively. Chlorogenic acid content was reduced by the enzymatic hydrolysis of the ester bond between caffeic acid and quinic acid, which induces the rise of the caffeic acid content (Zheng et al., 2009). Free sugars, pectin, and organic acids are needed to be eliminated, together with the isolation and enrichment of phenolics from the ACP. Amberlite XAD sorbents, which are reverse phase polymeric sorbents with a wide range of pH stability, are widely used for the isolation of aromatic and phenolics in the food industry (León-González and Pérez-Arribas, 2000). Moreover, the isolation and recovery of pectin and phenolic compounds from apple pomace using XAD sorbent have also been reported by Schieber et al. (2003). The diluted ACP solution applied to XAD-7 sorbent was washed out with neutral water and eluted with 95% ethanol. The adsorption and desorption rates of phenolics from ACP onto XAD-7 sorbent were investigated by HPLC analysis (Fig. 2 and Table 2). All tested phenolics except for chlorogenic acid (>86%), showed over 90% of adsorption and desorption rates onto the XAD-7 sorbents. These results indicate the usefulness of the XAD-7 sorbents to the isolation of fruits phenolics from more polar components, such as polysaccharides and organic acid. A similar pattern of phenolics isolation using XAD-4 sorbents has been reported by Miriam et al. (2009). Although acidic ethanol solution was used to enhance the recovery rates of honey phenolics (Michalkiewicz et al., 2008), 95% ethanol also showed good elution and was chosen for the present study (León-González and Pérez-Arribas, 2000) due to the high acid contents in ACS. Most polar compounds were not adsorbed in XAD-7 sorbent and eliminated during the adsorption-desorption process (Table 2). Briefly, Glycosylated phenolics in UAP were hydrolyzed to their aglycones in ACS through Viscozyme L reaction, concentrated to ACP, and purified by the adsorption- desorption process on XAD-7 sorbent, resulting in freeze-dried unripe apple antioxidant phenolics (AAP). The whole preparation process is shown in Fig. 1. TPC of AAP (498,723 mg/kg) was enriched to 771-folds, from UAP (647 mg/kg) through ACP (9,504 mg/kg). The significant increase of the individual content of phenolic, such as chlorogenic acid, caffeic acid, *p*-coumaric acid, phloridzin, and phloretin contents, also were observed (Table 1).

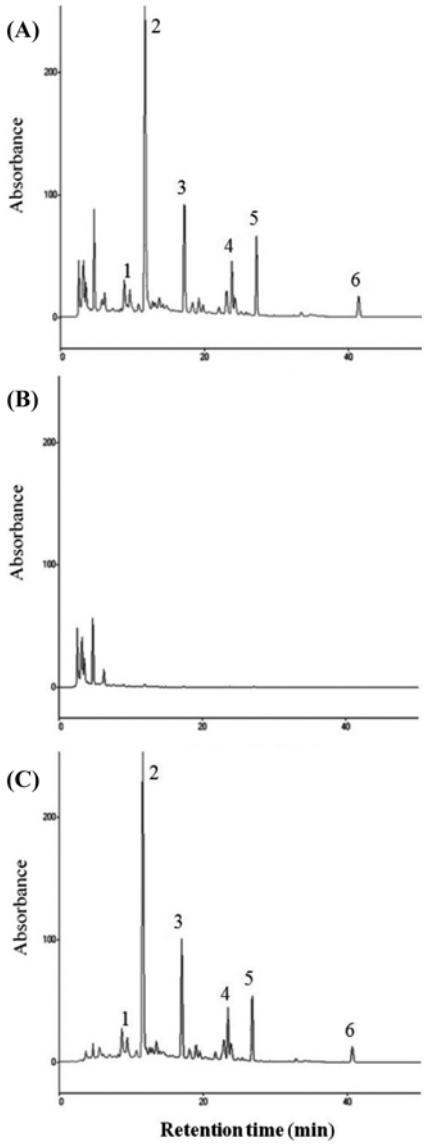

Fig. 2 HPLC chromatograms of unripe apple phenolics adsorption and desorption onto XAD-7. Apple crude phenolics (ACP) (A), adsorption (elution with H_2O) (B), desorption (elution with 95% ethanol) (C). 1, chlorogenic acid; 2, caffeic acid; 3, *p*-coumaric acid; 4, quercetin-3-glucoside; 5, phloridzin; 6, phloretin.

Table 2 Adsorption and desorption rates of unripe apple phenolics using XAD-7 sorbent (mg/kg)

Phenolics	ACS	Adsorption	Desorption
Total phenolic content	1105.24±3.07[a1)](100)[2)]	1062.64±2.8[a](96.14)	1054.13±2.1[a](95.38)
Chlorogenic acid	8.70±0.34[a](100)	7.75±0.28[b](88.97)	7.54±0.22[b](86.67)
Caffeic acid	43.13±0.45[a](100)	41.68±0.38[a](96.62)	40.57±0.33[b](94.10)
p-courmaric acid	20.70±0.24[a](100)	20.02±0.24[a](96.86)	19.69±0.20[a](95.12)
Phloridzin	27.41±0.11[a](100)	25.84±0.09[b](93.29)	25.27±0.08[b](92.19)
Phloretin	25.28±0.13[a](100)	24.36±0.10[a](96.32)	23.96±0.08[b](94.78)

[1)]The results are expressed as the mean ± SD. (n =3). Values followed by different lowercase letters within the same row are significantly different at p <0.05.
[2)]Sorption rate (%).

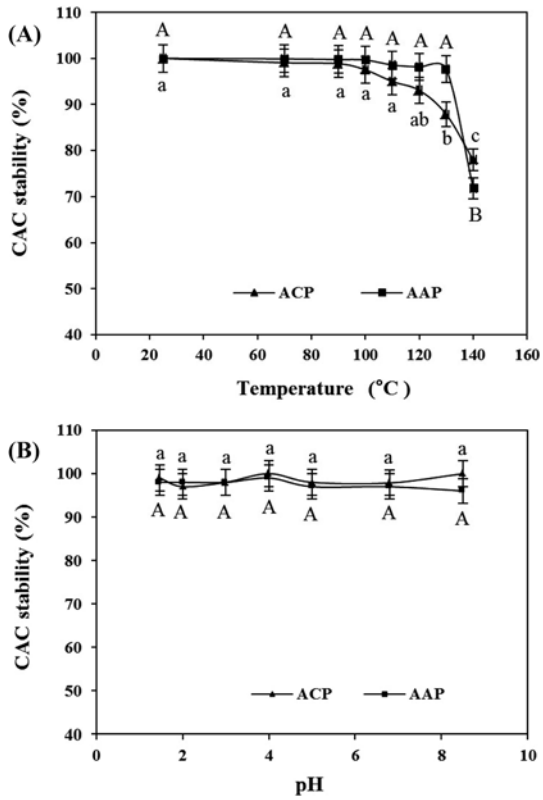

Fig. 3 Thermal stability (A) and pH stability (B) of the phenolics-rich products from unripe apples. ACP; apple crude phenolics, AAP; apple antioxidant phenolics. Bars represent standard deviations. Values followed by the same letters (lowercase; ACP, uppercase; AAP) are not significantly different(p <0.05).

The increased ratio of each content phenolic by HPLC determination was significantly higher than that of total phenolic content by spectrometry due to the high sensitivity. However, chlorogenic acid and phloridzin contents increase were lower due to the enzymatic hydrolysis to caffeic acid and phloretin, respectively (Zheng et al, 2009). AAP yield from UAP was so low value of 0.21%, but this value could be rise up to 8–9 times (1.68–1.89%) when dried raw material was used, although it is lower than that of catechin yield from green tea leaves (Vuong et al., 2011).

Table 3 Antioxidant activities of phenolics-rich products from unripe apples (mmol TE/g)

Product[1]	ORAC	DPPH	FRAP
UAP	13.68±0.20[2a]	3.27±0.02[a]	3.77±0.06[a]
ACS	23.84±0.34[b]	5.86±0.07[b]	5.42±0.05[b]
ACP	94.72±11.07[c]	34.71±0.25[c]	52.83±0.53[c]
AAP	8251.4±76.3[d]	2424.0±5.6[d]	2341.9±6.4[d]
Caffeic acid[3]	1.96±0.01	0.83±0.01	0.92±0.01

[1)]UAP: unripe apple, ACS: unripe apple crude solution, ACP: unripe apple crude phenolics, AAP: unripe apple antioxidant phenolics.
[2)]The results are expressed as the mean ± SD. (n =3).Values followed by different lowercase letters within the same column are significantly different at p <0.05.
[3)]Caffeic acid; 1 mmol.

Antioxidant activities of unripe apple phenolics products. Apples, especially in unripe ones, are considered as powerful antioxidants due to their ability to donate hydrogen or electrons and to form stable radical intermediates (Mohamed et al., 2001; Akiyama et al., 2005). Antioxidant activities of the plant extracts have been determined by several methods based on both the free radical scavenging and the oxidation-reduction mechanisms. Although none of the methods could represent the genuine antioxidant capacity, the spectrometric measurements of ORAC, DPPH radical scavenging and FRAP assays have been widely used due to their simplicity and representative results (Huang et al., 2005). Antioxidant activities of the phenolics-rich products, from UAP to AAP, are shown in Table 3, compared with standard antioxidants, caffeic acid (1 mmol). As the enrichment of the phenolics contents through the adsorption and desorption onto XAD-7 proceeds, the ORAC value of AAP increased up to about 90-fold of ACP, equivalent to 420 mmol of caffeic acid. DPPH and FRAP values of the AAP also increased up to about 70- and 45-fold, respectively. Considering the entire process including the Viscozyme L extraction and XAD-7 sorption, Antioxidant activities of the AAP increased approximately 600-fold, due to the significant increase of TPC (Table 1). Therefore, the Viscozyme L extraction coupled with XAD-7 sorption could be proposed to be a helpful process for the health benefit antioxidant production.

Thermal and pH stabilities. Conventional processing might result in significant losses of antioxidants in food products (Faller

and Fialho, 2009). Some phenolics could be fairly stable in high temperature processing (Vallejo et al., 2003; Faller and Fialho, 2009). In order to estimate the processing stabilities, ACP and AAP were kept at different temperatures and pH conditions for 1 h, and CAC were measured as an index for stability. When the storage temperature increased to 100°C, the stability of CAC in ACP slightly decreased, and was 75% at 140°C (Fig. 3). However, CAC of AAP was fairly stable at 120°C. In pH stability of CAC, both ACP and AAP showed more than 95% of stability at the entire tested pH range from 1.47 to 8.5. These results could validate that ACP and AAP could be utilized as potential antioxidant materials for the various types of processed foods accompanied by normal heat treatment without significant antioxidant loss.

Conclusion. Enrichment process of phenolics from unripe apples was studied and proposed. With the Viscozyme L extraction coupled with XAD-7 sorption process, AAP with 771-fold TPC was obtained from UAP. AAP could be utilized as potential antioxidant materials for the various heat- processed foods.

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