Chemical Composition and Antioxidant Activity of Crude Polysaccharide from Citron (*Citrus junos* Sieb. Ex TANAKA) Seed

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ABSTRACT: The crude polysaccharide (citron seed crude polysaccharide, CSCP) from citron (*Citrus junos* Sieb. Ex TANAKA) seed was obtained by water extraction and ethanol precipitation. The results of chemical composition analyses of the CSCP observed that neutral sugar content and uronic acid content were 27.70% and 14.70%, respectively. Mono-saccharide composition of the CSCP was as follows in the following order: galacturonic acid+glucuronic acid (14.70 mol%)> arabinose (12.85 mol%)> glucose (4.54 mol%)> galactose (4.32 mol%)> mannose (2.70 mol%)> fucose (1.68 mol%)> rhamnose (1.61 mol%). The total polyphenol contents of the CSCP was 30.42 µg/mL and 86.12 µg/mL at 1 mg/mL and 10 mg/mL, respectively ($P<0.001$). 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was higher at the concentration of the CSCP 10 mg/mL (47.12%) than that of the CSCP 1 mg/mL (14.02%), ($P<0.001$). The ABTS radical scavenging activity was higher than 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (14.33%) at the same concentration of the CSCP 10 mg/mL ($P<0.001$). Consequently, in this study, the crude polysaccharide from citron seed may be as a novel potential antioxidant.

Keywords: citron seed, crude polysaccharide, monosaccharide composition, antioxidant activity, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

INTRODUCTION

Citrus fruits are broadly grown around the world with a yearly production of about 102 million tons and these fruits are commonly used as fresh produce or in the juice form (1). The residues of juice-processing industry, such as peels, seeds, and pulps, which represent about 50% of the raw processed fruits, are a potential source of valuable by-products (2). Residues may pose pollution, disposal, and other related environmental problems due to microbial spoilage if not utilized (3). These citrus by-products can be a promising source for the food industry for their valuable nutritional properties. Citrus seeds are one of the by-products of citrus fruit processing. The importance of citrus seeds has been studied owing to the present diverse compounds, including polyphenols, tocopherols, phytosterols, and high amount of unsaturated fatty acids that can be useful for adding value to many products (2,4).

Plant polysaccharides from different sources have long been studied and widely used for a variety of purposes including food and medical industry (5). Polysaccharides also exhibit an array of biological activities that include

antioxidant, immune-modulatory, antitumor, gastrointestinal protection, and antidiabetic effects (6). Moreover, consumers are becoming more conscious of the nutritional value and safety of their food and ingredients. Preference for natural foods and food ingredients that are believed to be safer, healthier, and less subject to hazards is increasing compared to their synthetic counterparts (7). Polysaccharides have diverse biological activities and they are used in many healthcare products (8-10).

Citron (*Citrus junos* Sieb. Ex TANAKA) is a citrus fruit that is cultivated in Korea, China, and Japan. The citron fruit is commonly processed into beverages and herbal medicines due to its special flavor. Most parts of citron fruit are used, such as the peel, juice, and seeds (11) and citron seeds are 14~16% of total citron fresh weight (12). Citron seeds have limonoid contents and antioxidant activity (13). Limonoids derived from grapefruit seed are known to be antioxidants and they are highly valued. Citron seed can provide over 100 times the amount of limonoids compared to grapefruit seed in commercial production (13). Several *in vitro* studies have shown that limonoid components mediate the antioxidant properties of citrus. Reactive oxygen species (ROS) is believed to

Received 30 July 2018; Accepted 21 August 2018; Published online 31 December 2018

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be a factor in diseases with underlying cellular disorders (14). A previous study showed that citron seed extract using water or ethanol showed antioxidant activity and rich polyphenol and limonoids contents (15). Water is an economical and environmentally friendly solvent for the extraction of polysaccharides from raw material. Therefore, the aims of this study were to isolate water-soluble crude polysaccharides from citron seed (CSCP) and to evaluate the chemical composition (neutral and acidic sugars, protein, and monosaccharide composition analyses) and the antioxidant activities [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities].

MATERIALS AND METHODS

Preparation of the CSCP isolated from citron seed extraction powder (CSEP)

The citron seed in this study was cultivated at Goheung, Korea and purchased from the Ceil Food Company (Goheung, Korea). The seeds were washed twice using tap water and then dried using a conventional oven (Sanyo Electric Co., Ltd., Moriguchi, Japan) at 80°C overnight. Dried citron seeds were ground into powder (moisture content: 3.60%) with an electronic grinder (Hanil Electronics Corp., Wonju, Korea). The powder (100 g) of citron seed was extracted with 1 L of distilled water in a 40°C incubator (Sanyo Electric Co., Ltd.) for 8 h with stirring and then centrifuged at 6,000 rpm for 20 min. The supernatant was lyophilized CSEP. Lyophilized CSEP (7 g) was diluted with distilled water (100 mL). The sample was precipitated by the addition of four volumes of 95% ethanol. After centrifugation at 6,000 rpm for 30 min, the precipitate was then collected, and re-dissolved with distilled water. And then, the sample was dialyzed (12 kDa molecular weight cut off). The dialyzed sample was centrifuged at 6,000 rpm for 30 min and the supernatant was lyophilized, which was finally used as the CSCP isolated from the CSEP.

Chemical composition

Neutral sugar and uronic acid contents were analyzed by phenol-sulfuric acid (16) and m-hydroxybiphenyl (17) methods with galactose and galacturonic acid as the standards, respectively. 2-Keto-3-deoxy-D-manno-2-octulosonic acid (Kdo) content was determined by a modified thiobarbituric acid method (18), using Kdo as a reference. Protein content was determined by the Bradford's methods (19) using bovine serum albumin as the standard. Monosaccharide composition was analyzed by modified alditol acetate method of Jones and Albersheim (20) using gas chromatography (GC) (YL6000 Series, YL In-

strument Co., Ltd., Anyang, Korea) equipped with a SP-2380 capillary column (0.2 µm×0.25 mm×30 m; Supelco Inc., Bellefonte, PA, USA) and flame ionization detector. The temperature program of the GC was 60°C for 1 min, 60°C→220°C (30°C/min), 220°C for 12 min, 220°C→250°C (8°C/min), and 250°C for 15 min. The molar ratio of monosaccharides was calculated from the peak areas and response factors considering slope of each monosaccharide standard curve.

Total polyphenol contents

The total polyphenol content was determined using Folin-Ciocalteu method (21). Briefly, 0.79 mL of distilled water, 0.01 mL of appropriately diluted sample, and 0.05 mL of Folin-Ciocalteu reagent were added into test tubes and then mixed. Exactly 1 min later, 0.15 mL of 20% sodium carbonate was added. The mixture was then shaken and allowed to stand at room temperature for 2 h. The absorbance was measured at 750 nm, and the total polyphenol concentration was calculated from a calibration curve using gallic acid as a standard.

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined by the method of Cheung et al. (22). Briefly, 0.8 mL of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of an appropriately diluted sample. The mixture was shaken vigorously and allow to stand for 10 min in the dark. The decrease in absorbance was measured at 520 nm against a blank (without sample) in a spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{1 - A_s}{A_c} \times 100$$

where A_s is the absorbance in the presence of sample and A_c is the absorbance in the absence of sample, respectively.

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined by the method of Re et al. (23). Briefly, The ABTS radical was generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and then allowing the mixture to stand overnight in the dark at room temperature. The ABTS radical solution was then diluted with distilled water to obtain an absorbance of 1.4~1.5 at 414 nm. And then, 1 mL diluted ABTS radical solution was added to a 50 µL of the sample. After 1 h, the absorbance was measured at 414 nm. The ABTS radical scavenging activity was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{1 - A_s}{A_c} \times 100$$

where A_s is the absorbance in the presence of sample and A_c is the absorbance in the absence of sample, respectively.

Statistical analysis

All statistical analyses were conducted using the Statistical Package for Social Sciences, version 12.0 (SPSS Inc., Chicago, IL, USA). Differences among the samples were evaluated statistically by *t*-test or one-way analysis of variance and Duncan's multiple tests. The level of statistical significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS & DISCUSSION

Preparation of the CSCP and chemical composition

Polysaccharides are the natural polymers formed from various monosaccharides units and their derivatives. They are widely distributed in microorganisms, animal cells, and plant cells. Due to their strong antioxidant activities and free radical scavenging abilities, many polysaccharides have been explored for development into safe and effective medicines (24,25). Therefore, in this study, the CSCP was isolated from the CSEP using ethanol precipitation and the yield was 14% (Table 1). The results of chemical composition analyses and GC chromatogram of the CSCP are shown in Table 2 and Fig. 1, respectively. Neutral sugar and uronic acid contents were 27.70%

Table 1. Yield of the CSCP prepared from the CSEP

CSEP (g)	CSCP (g)	Yield (%)
7.00	0.98	14.00

CSEP, citrons seed extraction powder; CSCP, citron seed crude polysaccharide.

and 14.70%, respectively. Protein content was higher at 57.60% compared to sugar contents. Monosaccharide composition analysis revealed that the CSCP showed high contents of arabinose (12.85 mol%) and galacturonic acid+glucuronic acid (14.70 mol%) and then it was in the following order: glucose (4.54 mol%) > galactose (4.32 mol%) > mannose (2.70 mol%) > fucose (1.68 mol%) > rhamnose (1.61 mol%).

Monosaccharide composition of plant polysaccharides are varied. Polysaccharide isolated from *Camellia oleifera* Abel. seed was composed of xylose, glucuronic acid, galactosamine, and mannose, and their monosaccharide molar composition ratio was 10.9:4.4:2.6:1.8 (26). Wang et al. (27) reported that three crude polysaccharide fractions were obtained from *Citrus aurantium* L. by cold water, hot water, and 1 M NaOH. Among them, *Citrus aurantium* L. hot water fraction (CALB) showed the highest neutral sugar content (81.35%) and the lowest uronic acid content (48.76%), and its protein content was 17.01%. And

Table 2. The chemical composition of the citron seed crude polysaccharide

Chemical composition (%)	
Neutral sugar	27.7±6.7 ¹⁾
Uronic acid	14.7±0.8
Kdo-like material	0
Protein	57.6±3.5
Monosaccharide composition (mol%)	
Arabinose	12.85±1.0
Galactose	4.32±0.5
Rhamnose	1.61±0.0
Xylose	0
Glucose	4.54±0.3
Mannose	2.70±0.1
Fucose	1.68±0.2
Galacturonic acid+glucuronic acid	14.7±0.8
Kdo	0

¹⁾Data are expressed as mean±standard deviation. Kdo, 2-keto-3-deoxy-D-manno-2-octulosonic acid.

Fig. 1. Gas chromatography chromatogram of the standards (A) and the citron seed crude polysaccharide (B) for analysis of monosaccharide composition.

then, CALB purified ion exchange and gel-filtration chromatography in four groups of CALB-1, CALB-2, CALB-3, and CALB-4. CALB-1 (42.2 mol%) and CALB-2 (49.4 mol%) were mainly composed of galacturonic acid. CALB-3 was mainly composed of galactose (45.8 mol%) and arabinose (37.8 mol%) and CALB-4 was mainly composed of galactose and arabinose, and their corresponding molar percentages (mol%) were 21.7% and 41.7%, respectively.

Total polyphenol content and antioxidant activities

Citrus is one of the most abundant crops in the world and citrus seeds are by-products of citrus fruit processing (28). Recent epidemiological studies have strongly suggested that consumption of certain plant polysaccharides such as seeds, leaves, fruits, and roots may reduce the risk of chronic diseases related to oxidative stress on account of their antioxidant activity and promote general health benefits (29). Therefore, the CSCP isolated from the CSEP was examined for total polyphenol content and antioxidant activities using ABTS and DPPH radicals. Phenolics are aromatic secondary plant metabolites and called high level antioxidants because of their ability to scavenge free radicals and ROS such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and the hydroxyl radical ($HO^{\cdot}$) (30,31). Natural polyphenols have been recognized to play a determining role in many degenerative diseases, including cancer and atherosclerosis (32). Total polyphenol content is commonly quantified using the Folin-Ciocalceau method (33). This method is ideal not only for separating phenolic compounds but also their quantification (34). In this study, total polyphenol content of the CSCP was evaluated using Folin-Ciocalceau method and the result are shown in Fig. 2. The total polyphenol content of the CSCP was 30.42 $\mu\text{g/mL}$ and 86.12 $\mu\text{g/mL}$ at the concentration of 1 mg/mL and 10 mg/mL, respectively ($P < 0.001$). The results of ABTS and DPPH radical scavenging activities of the CSCP are shown in Fig. 3. The ABTS radical scavenging activity of the CSCP was higher at the

Fig. 2. Total polyphenol content of the citron seed crude polysaccharide. ***Significantly different at $P < 0.001$ by t -test.

concentration of 10 mg/mL (47.12%) than that of 1 mg/mL (14.02%), ($P < 0.001$). The DPPH radical scavenging activity was not significantly different between 1 mg/mL (10.19%) and 10 mg/mL (14.33%), ($P > 0.05$). Some researchers reported that the correlation between the total polyphenol content and the ABTS assay was stronger than the DPPH assay (35). In this study, ABTS radical scavenging activity showed significant differences ($P < 0.001$) between low (30.42 $\mu\text{g/mL}$) and high total polyphenol contents (86.12 $\mu\text{g/mL}$), whereas DPPH radical scavenging activity did not show significant differences ($P > 0.05$).

Shi et al. (36) isolated the polysaccharides of peony seed dreg, which was sequentially extracted with hot buffer, chelating agent, diluted alkali, and concentrated alkali, and the corresponding products were assigned as HBSS, CHSS, DASS, and CASS, respectively. DPPH measurement of free radical scavenging activity of four fractions (HBSS, CHSS, DASS, and CASS) showed a dose-dependent change in scavenging ability. At a concentration of 5.0 mg/mL, the DPPH free radical scavenging activity for HBSS, CHSS, DASS, and CASS were 78.86%, 71.15%, 79.73%, and 92.95, respectively. The ABTS radical scavenging activities of all fractions increased in a dose-dependent manner. At the concentration of 2 mg/mL, CASS almost showed the same scavenging activity (100%) as ascorbic acid. Luo et al. (37) isolated crude polysaccharide (PMTP) from *Polygonum multiflorum* Thunb using hot water extraction followed by ethanol extraction. The PMTP exhibited high ABTS radical scavenging activity at the concentration of 3.0 mg/mL (96.45%) and 5.0 mg/mL (97.54%). And DPPH radical scavenging effect of the PMTP was also high (87.15%) at the concentration of 5.0 mg/mL.

Many factors affect antioxidant activities of polysaccharides, including monosaccharide composition, molecular mass, glycosyl residues, and chain conformation (38). *Spirulina* blue polysaccharide consisted of mannose, glu-

Fig. 3. Antioxidant activities of the citron seed crude polysaccharide. ABTS; 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity, DPPH; 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity. Different letters (a,b) mean significant differences among samples at $P < 0.001$ by ANOVA test.

curonic acid, galactose, xylose, and rhamnose. It could effectively remove hydroxyl free radicals and ABTS radicals (39). The antioxidant activities *in vitro* of the polysaccharides from *Corbicula fluminea* were studied. It was found that the purified polysaccharides had a higher antioxidant activities than the crude polysaccharides (40). Four purified polysaccharides (CALB-1, CALB-2, CALB-3, and CALB-4) were isolated from CALB (*Citrus aurantium* L.), among which CALB-3 showed the strongest antioxidant activity against DPPH and hydroxyl *in vitro*. Analysis of the monosaccharide composition of four purified polysaccharides demonstrated that CALB-3 and CALB-4 mainly consisted of neutral sugars: mannose, rhamnose, galactose, and arabinose (92.2% and 83.7%, respectively), with small amounts of the acidic sugars: glucuronic acid and galacturonic acid (7.8% and 16.3%, respectively). CALB-3 and CALB-4 exhibited greater antioxidant effects than CALB-1 and CALB-2, and this may be due to their higher neutral sugar content (27). The DPPH radical scavenging effects of the three groups of purified polysaccharides (CALB-1, CALB-2, and CALB-3) were evidently in a dose-dependent manner (27). In this study, the CSCP showed a higher neutral sugars composition, including arabinose, glucose, galactose, mannose, rhamnose, and fucose than acidic sugar, including galacturonic acid and glucuronic acid. The ABTS radical scavenging activity of the CSCP was higher than the DPPH radical scavenging activity at the concentration of CSCP 10 mg/mL ($P < 0.001$).

Nowadays, plant derived food supplements are replacing the synthetic food supplements as consumers are becoming increasingly aware of healthy nutrition and diet related health problems. The emerging demand for natural ingredients has encouraged the research for identifying newer and cost effective plant-derived nutraceuticals, which can replace the expensive synthetic food supplements in near future. The exploitation of citrus by-products as a source of functional compounds and its use in pharmaceutical formulations has now become a promising field (41). In this study, the CSCP isolated as citrus by-products possessed moderate ABTS radical scavenging effect as a novel antioxidant candidate. However, more research and further investigations are required to understand crude polysaccharide from citron seed as bioactive materials and therapeutic potential the acceptability in the food and medicine industries, including the mechanism of prevention of chronic diseases related to oxidative stress. Nonetheless, these results provided a basis and direction for further studying the antioxidant activities of citron seed polysaccharides.

AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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