Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Hye-Kyung Seo[a]; Seung-Cheol Lee[a]
[a] Department of Food Science and Biotechnology, Kyungnam University, Masan, Korea

Online publication date: 21 January 2010

To cite this Article Seo, Hye-Kyung and Lee, Seung-Cheol(2010) 'Antioxidant Activity of Subcritical Water Extracts from Chaga Mushroom (*Inonotus obliquus*)', Separation Science and Technology, 45: 2, 198 — 203
To link to this Article: DOI: 10.1080/01496390903423899
URL: http://dx.doi.org/10.1080/01496390903423899

Separation Science and Technology, 45: 198–203, 2010

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390903423899

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Antioxidant Activity of Subcritical Water Extracts from Chaga Mushroom (*Inonotus obliquus*)

Hye-Kyung Seo and Seung-Cheol Lee
Department of Food Science and Biotechnology, Kyungnam University, Masan, Korea

The subcritical water (SCW) extraction of Chaga mushroom (CM) was carried out at various temperatures (50, 100, 150, 200, 250, and 300°C) and times (10, 30, and 60 min), and then antioxidant activities of the SCW extracts were evaluated by determining 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, reducing power (RP), superoxide dismutase (SOD)-like activity, and total phenol content (TPC). The DPPH and ABTS radical scavenging activities, and the SOD-like activity of the extracts increased with elevated temperatures and times. For example, DPPH and ABTS radical scavenging activities, and SOD-like activity of the extracts at 250°C for 60 min were 72.5, 97.8, and 92.3%, respectively, while those at 50°C for 60 min were 63.2, 14.4, and 22.6%, respectively. However, the activities decreased at 300°C. The highest TPC and RP were found at 250°C for 30 min with values of 10.724 mg/mL and 1.063 optical density, respectively. These results indicate that SCW extraction was significantly effective on the increase of antioxidant activity of the CM.

Keywords antioxidant activity; Chaga mushroom; extraction; subcritical water

INTRODUCTION

The subcritical water (SCW) is hot water under pressure sufficient to maintain its liquid state (critical point of water, 22.4 MPa and 374°C). SCW has unique characteristics such as high density, high reactivity, good solubility for a series of organic compounds having relatively low molecular weights, and the ability to hydrolyze ester and ether bonds contained in polymer chains. Based on these features, SCW has been used to extract environmental pollutants from contaminated soils, sediments, and sludge (1–3). Recently, several attempts for extracting valuable materials with SCW from bio-resources have been reported (4–8). For example, SCW was used to extract nutraceuticals with antioxidant activities from oregano (5), flavor compounds from rosemary (7), and polyphenolics from *Pinus taiwanensis* and *Pinus morrisonicola* (8).

A Chaga mushroom (*Inonotus obliquus* or *Fuscosporia obliqua*) is white rot fungus that belongs to the Hymenochaetaceae family of Basidomycetes. It is a black parasitic fungus which grows on living trunks of the mature birch, and is mainly found at latitudes of 45°–50° (9). A Chaga mushroom has been used in folk medicine for treating cancer in Russia, Western Siberia, Asia, and North America (10). It has been known that a Chaga mushroom contains many polyphenolic compounds and has various biological activities, including anti-bacterial (11), hepatoprotective (12), and anti-tumor properties (13–15). Cui et al. (16) reported that polyphenolic compounds extracted from the Chaga mushroom showed significant antioxidant activity, and Kim et al. (14) identified many polyphenolic compounds, triterpenoids, and steroids such as lanosterol, inotodiol, trametenolic acids, and ergosterol peroxides from Chaga mushroom. The extracts from Chaga mushroom by boiling water has been mostly prepared and applied as folk medicine. In this study, we prepared SCW extracts from Chaga mushroom at several different temperatures (50, 100, 150, 200, 250, and 300°C) during three different time periods (10, 30, and 60 min), and then evaluated antioxidant activities of the extracts to determine the optimum condition for SCW extraction from Chaga mushroom.

EXPERIMENTAL

Materials and Reagents

The Chaga mushrooms were purchased from Kumkang Pharm. Co., Ltd. (Masan, Korea). Ferric chloride, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablets, trichloroacetic acid (TCA), and superoxide dismutase (SOD) assay kit was from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu reagent was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The water used in this study was prepared with a super purity water system (Purite Ltd., Oxon, U.K.) with a resistance of >17.5 MΩ cm.

Received 29 June 2009; accepted 30 September 2009.
Address correspondence to Seung-Cheol Lee, , Department of Food Science and Biotechnology, Kyungnam University, Masan 631-701, Korea. Tel.: 82-55-249-2995; Fax: 82-55-249-2995. E-mail: sclee@kyungnam.ac.kr

SCW Extraction

Reaction vessels of stainless steel tube for SCW extraction were prepared with a length of 14 cm (inner volume 18.57 mL). Chaga mushroom were ground in a mill and the powder with particle diameter smaller than 710 μm (25-mesh) was used in the following extraction preparation. A Chaga mushroom (0.02 g) and 10 mL of water were placed in the vessel. After being tightly closed with a stainless steel cap, the vessel was placed in a muffle furnace (Daeil Engineering, Seoul, Korea). SCW extractions from Chaga mushroom were carried out at 50, 100, 150 and 300°C (0.002 to 5 MPa) for 10, 30, and 60 min, respectively. Then the vessels were taken, out of the furnace and cooled at room temperatures. The SCW extracts were filtered through filter paper (Whatman No. 3, Whatman, UK) and the filterate was stored in a deep freezer (Operon Co., Seoul, Korea) at −70°C for further experiments.

DPPH Radical Scavenging Activity (RSA)

The DPPH radical scavenging activity (RSA) of the SCW extracts from Chaga mushroom was determined according to the method of Lee et al. (17). After mixing 0.1 mL of each SCW extract with 0.9 mL of 0.041 mM DPPH in ethanol for 30 min, the absorbance of the sample was measured at 517 nm by a spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). Radical scavenging activity was expressed as percentage according to the following formula:

$$\%\ \text{DPPH radical scavenging activity} = [1 - (A_{sample}/A_{control})] \times 100$$

ABTS Radical Scavenging Activity (RSA)

The ABTS RSA was determined according to the method of Muller (18). The SCW extract (0.1 mL), potassium phosphate buffer (0.1 mL, 0.1 M, pH 5.0) and hydrogen peroxide (20 μL, 10 mM) were mixed and incubated at 37°C for 5 min. After preincubation, ABTS (30 μL, 1.25 mM, in 0.05 M phosphate-citrate buffer, pH 5.0) and peroxidase (30 μL, 1 unit/mL) were added to the mixture and then it was incubated at 37°C for 10 min. The absorbance level was obtained with a multiplate reader (Sunrise RC/TS/TS Color-TC/TW/BC/6Filter, Tecan Austria GmbH.) at 405 nm.

$$\%\ \text{Hydrogen peroxide inhibition activity} = [(1 - A_{sample}/A_{control})] \times 100$$

Reducing Power (RP)

The RP of the SCW extract was determined according to the method of Meir et al. (19). After 5-fold diluting the SCW extract with water, 1 mL of the diluted SCW extract, 200 mM sodium phosphate buffer (1 mL, pH 6.6) and 1% potassium ferricyanide (1 mL) were mixed and incubated at 50°C for 20 min. Then, 10% TCA (1 mL) was added to the mixture and centrifuged at 13,400 × g for 5 min. The supernatant (1 mL) was mixed with distilled water (1 mL) and 0.1% ferric chloride (0.1 mL), and then the absorbance was measured at 700 nm.

Superoxide Dismutase (SOD)-like Activity

SOD-like activity of the SCW extracts was determined by SOD assay kit according to the manuals. In a 96-well plate, 20 μL of sample solution was added to the sample and blank 2 well, and then 20 μL of double-distilled water was added to blank 1 and blank 3 well. Two hundred μL of WST (water-soluble tetrazolium salt) working solution was added to each well, and 20 μL of dilution buffer was added to the blank 2 and the blank 3 well. Twenty μL of enzyme working solution was added to each sample and the blank 1 well and then mixed thoroughly. The plate was incubated at 37°C for 20 min and then absorbance for the plate was measured by the multiplate reader at 450 nm. SOD-like activity was calculated according to the following equation:

$$\%\ \text{SOD-like activity} = [(A_{blank1} - A_{blank3}) - (A_{Sample} - A_{blank2})]/(A_{blank1} - A_{blank3})] \times 100$$

Total Phenolic Contents (TPCs)

The TPCs of the Chaga mushroom extracts were determined according to the method of Gutfinger (20). After the extracts were diluted with 20-fold in water, 1 mL of the diluted extract was mixed with 1 mL of 2% Na_2CO_3 and 1 mL of 50% Folin-Ciocalteu reagent, and centrifuged at 13,400 × g for 5 min. After 30 min incubation at room temperature, the absorbance was measured at 750 nm. The TPCs were expressed as gallic acid equivalents.

Statistical Analyses

All measurements were performed in triplicate, and analyses of variance were conducted by the General Linear Model procedure using SAS 6.12 software (21). Student-Newman-Keul's multiple range tests were used to test significant differences between the mean values for the treatments ($P < 0.05$).

RESULTS AND DISCUSSION

DPPH RSA of SCW Extracts from Chaga Mushroom

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples (22,23). DPPH significantly decreased depending on the exposure to proton radical scavengers (24). The DPPH RSA of the SCW extracts from Chaga mushroom and ascorbic acid (positive control) were expressed in Table 1. As shown in Table 1, the DPPH

TABLE 1
Effects of subcritical water extraction on DPPH radical scavenging activity from Chaga mushroom (*Inonotus obliquus*)

Temperature (°C)	Time (min) 10	30	60	L-ascorbic acid (100 μg/mL) (%)
50°C	31.8 ± 0.1^{ex}	40.5 ± 0.7^{dy}	63.2 ± 0.5^{cz}	94.4 ± 0.3
100°C	42.5 ± 0.2^{dx}	55.5 ± 0.2^{bcy}	60.8 ± 0.2^{dz}	
150°C	50.9 ± 0.3^{cx}	54.1 ± 0.1^{cy}	54.9 ± 0.1^{ez}	
200°C	57.6 ± 0.1^{ay}	56.9 ± 1.2^{by}	67.3 ± 0.8^{bz}	
250°C	55.2 ± 0.4^{bx}	64.9 ± 0.6^{ay}	72.5 ± 1.0^{az}	
300°C	55.0 ± 0.9^{by}	64.2 ± 1.4^{az}	52.7 ± 0.1^{fx}	

$^{a-e}$Different letters within a row are significantly different ($P < 0.05$), n = 3.
$^{x-z}$Different letters within each extract are significantly different ($P < 0.05$), n = 3.
Twenty mg of Chaga mushroom was extracted by 10 mL of subcritical water at given temperature for given time.

RSA of the SCW extracts significantly ($P < 0.05$) increased as extraction temperature and times increased. The maximum DPPH RSA (72.5%) was obtained when the SCW extraction from Chaga mushroom was performed at 250°C for 60 min. The SCW extraction was able to selectively obtain antioxidant compounds from rosemary (25), and Baek et al. (26) reported that the DPPH RSA of licorice root increased in SCW extracts prepared at 200°C for 60 min or at 300°C for more than 30 min. Our results suggest that antioxidant compounds in a Chaga mushroom could be extracted with SCW.

The DPPH RSA of L-ascorbic acid (100 μg/mL) was 94.4%, which was higher than the highest value of the SCW extracts from Chaga mushroom. When considering the amount of antioxidant compounds (2000 μg/mL) from Chaga mushroom, L-ascorbic acid had much higher DPPH RSA than that of Chaga mushroom. This might be due to the fact that L-ascorbic acid was a purified single compound while the SCW extracts was crude antioxidant compounds. On the other hand, Cui et al. (16) reported that the polyphenolic fraction of 80% ethanol extract from Chaga mushroom showed more than 7 times of DPPH radical scavenging activity than water fraction. They also described that 50 μg/mL of the water fraction of Chaga mushroom exhibited $11.0 \pm 0.5\%$ of DPPH radical scavenging activity; however, it is difficult to compare with our data directly because our SCW extract was crude one, while the water fraction of Cui et al. (16) was sequentially fractionated one excluding many compounds.

ABTS RSA of SCW Extracts From Chaga Mushroom

ABTS is a peroxidase substrate which, when oxidized in the presence of H_2O_2 in a typical peroxidative reaction, generates a metastable radical with a characteristic absorption spectrum and an absorption maximum of 414 nm (27). The $ABTS^+$ radicals are scavenged by antioxidants via

TABLE 2
Effects of subcritical water extraction on ABTS from Chaga mushroom (*Inonotus obliquus*)

Temperature (°C)	Time (min) 10	30	60	L-ascorbic acid (100 μg/mL) (%)
50°C	7.1 ± 0.1^{ex}	11.7 ± 0.4^{fy}	14.4 ± 0.2^{fz}	95.6 ± 0.5
100°C	6.1 ± 0.2^{fx}	30.1 ± 0.2^{dy}	33.5 ± 0.1^{ez}	
150°C	14.5 ± 0.5^{dx}	24.6 ± 0.5^{ey}	44.2 ± 0.1^{dz}	
200°C	25.2 ± 0.4^{cx}	69.0 ± 4.7^{by}	93.2 ± 0.1^{bz}	
250°C	26.6 ± 0.6^{by}	96.9 ± 0.5^{az}	97.8 ± 0.3^{az}	
300°C	39.1 ± 0.6^{ax}	44.2 ± 2.6^{cy}	60.9 ± 0.1^{cz}	

$^{a-d}$Different letters within a row are significantly different ($P < 0.05$), n = 3.
$^{x-z}$Different letters within each extract are significantly different ($P < 0.05$), n = 3.

TABLE 3
Effects of subcritical water extraction on reducing power from Chaga mushroom (*Inonotus obliquus*)

Temperature (°C)	Time (min) 10	30	60	L-ascorbic acid (100 μg/mL) (O.D)
50°C	0.149 ± 0.003^{fx}	0.178 ± 0.003^{fy}	0.249 ± 0.003^{fz}	1.048 ± 0.001
100°C	0.169 ± 0.003^{dx}	0.246 ± 0.002^{ey}	0.332 ± 0.003^{ez}	
150°C	0.156 ± 0.001^{ex}	0.287 ± 0.003^{dy}	0.561 ± 0.001^{dz}	
200°C	0.272 ± 0.003^{cx}	0.655 ± 0.001^{cy}	0.950 ± 0.002^{bz}	
250°C	0.396 ± 0.001^{bx}	1.063 ± 0.010^{az}	0.966 ± 0.001^{ay}	
300°C	0.622 ± 0.001^{ax}	0.669 ± 0.001^{by}	0.683 ± 0.008^{cz}	

$^{a-d}$Different letters within a row are significantly different ($P < 0.05$), n = 3.
$^{x-z}$Different letters within each extract are significantly different ($P < 0.05$), n = 3.

the mechanism of electron-/hydrogen-donation and are assessed by measuring the decrease in absorption at 405 nm. The ABTS RSA of the SCW extracts from Chaga mushroom and ascorbic acid were shown at Table 2. Like DPPH RSA, the ABTS RSA increased with elevated extraction temperatures and times. The highest ABTS RSA of 97.8% was found in the SCW extracts obtained at 250°C for 60 min, which was 6 times higher than that (14.4%) of the extract obtained at 50°C for 60 min. The ABTS RSA of L-ascorbic acid was 95.6%, which was lower than the highest value (97.8%) of the SCW extract. These results indicate that SCW was an effective solvent on the extraction of the compounds with ABTS RSA from the mushroom, exhibiting strong ABTS RSA.

RP of SCW Extracts from Chaga Mushroom

The antioxidant ability of certain compounds is associated with their RP (28), thus the RP may serve as a significant indicator of potential antioxidant activity (19). RP was determined by measuring the reduction of the Fe^{3+} form of Fe^{3+}/ferricyanide complexes to the ferrous (Fe^{2+}) form. Table 3 shows the effect of extraction temperatures and times on the RP of SCW extracts. RP was measured with 5-fold dilution because SCW extracts had too high RP to measure. The highest RP of 1.063, which was similar with L-ascorbic acid (1.048), was found in the SCW extracts obtained at 250°C for 30 min, and relatively higher RP was measured in extracts obtained at 200°C for 60 min or at 250°C for more than 30 min. These results indicate that SCW seems to be the promising solvent to extract the antioxidant components with RP from Chaga mushroom.

SOD-like Activity of SCW Extracts from Chaga Mushroom

SOD is the only eukaryotic enzymes known to remove toxic superoxide anions. Like RP, the SCW extracts were diluted with 5-fold to measure SOD-like activity because the SCW extracts had too high SOD-like activity to measure. As shown in Table 4, the extraction temperatures and times significantly ($P < 0.05$) affected on the SOD-like activity of the extracts from Chaga mushroom. For example, the SOD-like activity increased from 22.6 to 92.3% as the extraction temperatures increased from 50°C to 250°C

TABLE 4
Effects of subcritical water extraction on SOD activity from Chaga mushroom (*Inonotus obliquus*)

Temperature (°C)	Time (min) 10	30	60	L-ascorbic acid (100 μg/mL) (%)
50°C	5.5 ± 0.4^{dy}	4.6 ± 0.6^{fy}	22.6 ± 1.6^{fz}	21.8 ± 1.5
100°C	5.4 ± 1.1^{dx}	8.3 ± 1.6^{ey}	29.4 ± 1.6^{ez}	
150°C	7.9 ± 4.5^{dx}	28.7 ± 1.8^{dy}	53.3 ± 1.2^{dz}	
200°C	21.3 ± 1.4^{cx}	61.6 ± 0.7^{cy}	74.8 ± 1.0^{cz}	
250°C	29.5 ± 1.9^{bx}	77.2 ± 1.6^{by}	92.3 ± 0.9^{az}	
300°C	53.1 ± 2.5^{ax}	89.6 ± 0.7^{az}	82.3 ± 0.3^{by}	

$^{a-d}$Different letters within a row are significantly different ($P < 0.05$), n = 3.
$^{x-z}$Different letters within each extract are significantly different ($P < 0.05$), n = 3.

for 60 min. The highest SOD-like activity (92.3%) of the SCW extract was 4 times higher than that of L-ascorbic acid (21.8%).

TPCs of SCW Extracts from Chaga Mushroom

Phenolic compounds are the most active antioxidant derivatives in plants (29), and are known to act as antioxidants not only because of their ability to donate hydrogen or electrons, but also because they are stable radical intermediates (30). The TPCs of SCW extracts from Chaga mushroom were shown in Table 5. The TPCs of the extracts were also significantly ($P < 0.05$) affected by extraction temperatures and times. The highest TPC of 10.72 mg/mL was measured in the SCW extracts obtained at 250°C for 30 min, which was 18 times more than that (0.61 mg/mL) of the extract obtained at 50°C for 10 min. Our previous studies (31–34) showed that heat treatment converted insoluble phenolic compounds to soluble phenolics. These indicated that simple heat treatment could cleave the covalently bound phenolic compounds. The SCW extraction at high temperature also increased the amount of phenolic compounds of oregano leaves (6) and rosemary (8). These results suggest that SCW is an efficient solvent to extract phenolic compounds from Chaga mushroom.

Correlation between TPC and Antioxidant Activities of the SCW Extracts

To evaluate a primary contributing effect of TPCs to the total antioxidant activities of SCW extracts from Chaga mushroom, the linear correlations were analyzed with the four antioxidant assays (DPPH RSA, ABTS RSA, reducing power, and SOD-like activity) and TPCs as GAE unit. The calculated coefficients of correlations between TPCs and antioxidant activities of the SCW extracts from Chaga mushroom are shown in Table 6. Good linear correlations ($r = 0.90$ and 0.93, respectively) were found between ABTS and TPC, and RP and TPC, while a correlation between DPPH RSA and TPCs was weak ($r = 0.62$). The low correlation between TPCs and DPPH radical scavenging activity indicates that the other bioactive components such as polysaccharides, triterpenoids, and steroids from Chaga mushroom may play important roles in DPPH radical scavenging activity (16). Among the antioxidant activities, the highest correlation ($r = 0.97$) was found between ABTS and RP.

Because polyphenolic substances are generally known to be closely related to antioxidant activity and RP (35), the antioxidant and RP of the SCW extracts from Chaga mushroom could be mainly due to its phenolic compounds. On the other hand, β-glucan has been reported as a primary component responsible for physiological functions of Chaga mushroom (9,15), and it also represents significant antioxidant activity (36). In this study, we attempted to determine the amount of β-glucan of the SCW extracts from Chaga mushroom, however, it was difficult to obtain exact values because of the scale of our experiment. β-Glucan might play an important role in antioxidant activity of the SCW extracts from Chaga mushroom.

TABLE 5
Effects of subcritical water extraction on total phenol content from Chaga mushroom (*Inonotus obliquus*)

Temperature (°C)	Time (min)		
	10	30	60
50°C	0.61 ± 0.05^{fx}	0.90 ± 0.05^{fy}	1.07 ± 0.02^{ez}
100°C	0.86 ± 0.01^{ex}	1.58 ± 0.06^{ey}	2.51 ± 0.11^{dz}
150°C	1.04 ± 0.01^{dx}	2.48 ± 0.02^{dy}	5.54 ± 0.02^{bz}
200°C	1.89 ± 0.01^{cx}	6.77 ± 0.05^{by}	9.54 ± 0.01^{az}
250°C	2.90 ± 0.06^{bx}	10.72 ± 0.06^{az}	5.53 ± 0.04^{by}
300°C	5.59 ± 0.01^{az}	4.85 ± 0.04^{cy}	3.60 ± 0.02^{cx}

[a–d]Different letters within a row are significantly different ($P < 0.05$), $n = 3$.

[x–z]Different letters within each extract are significantly different ($P < 0.05$), $n = 3$.

TABLE 6
Correlation analysis between total phenol content and four antioxidant activities of SCW extracts from Chaga mushroom (*Inonotus obliquus*)

Factor	TPC	DPPH-RSA	ABTS-RSA	Reducing power	SOD
TPC	1.00	0.62	0.90	0.93	0.80
DPPH-RSA	–	1.00	0.72	0.70	0.69
ABTS-RSA	–	–	1.00	0.97	0.87
Reducing power	–	–	–	1.00	0.93
SOD	–	–	–	–	1.00

Correlation is significant at the $P < 0.05$.

CONCLUSION

The SCW extraction from Chaga mushroom liberated phenolic compounds from it, resulting in increasing the levels of active compounds in the extracts. Higher extraction temperatures and times were more effective on the production of the antioxidative substances. This study suggests that SCW extraction can be used as a tool to increase the antioxidant activities in Chaga mushroom.

ACKNOWLEDGEMENT

This study is supported by a research grant from Kyungnam University, Korea, in 2008.

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