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Effect of steam treatment on soluble phenolic content and antioxidant activity of the Chaga mushroom (*Inonotus obliquus*)

Hyun Kyoung Ju [a], Ha Wook Chung [a], Soon-Sun Hong [b], Jeong Hill Park [a], Jeongmi Lee [c,*], Sung Won Kwon [a,*]

[a] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151742, Republic of Korea
[b] School of Medicine, Inha University, Incheon 400712, Republic of Korea
[c] Department of Pharmacology, The University of Texas Southwestern Medical Center at Dallas, Texas 75390, USA

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ABSTRACT

The effect of steam treatment on free phenolic acids in Chaga mushrooms (*Inonotus obliquus*) was investigated. Untreated and steam-treated (120 °C, 3 h) samples of *I. obliquus* were extracted with organic solvents and free phenolic acid-containing fractions were isolated. Free phenolic acids were determined by LC/PDA (liquid chromatography/photodiode array), ESI LC/MS (electrospray ionisation liquid chromatography/mass spectrometry), and GC/MS (gas chromatography/mass spectrometry). After the steam treatment, the soluble phenolic content determined by modified Folin–Ciocalteu method was increased and antioxidant activity was enhanced, as confirmed by a DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity assay. The amounts of vanillic acid, protocatechuic acid, syringic acid, and 2,5-dihydroxyterephthalic acid were increased significantly as the result of the steam treatment, suggesting that the liberation of low molecular weight free phenolics was enhanced by the steaming process. Consequently, the radical scavenging activity was also significantly enhanced by free phenolics produced using this method.

1. Introduction

Inonotus obliquus, also known as the Chaga Mushroom (CM), is a fungus that belongs to the Hymenochaetaceae family of Basidiomycetes. It grows on birch or other trees in the birch forests of Russia, Korea, Eastern Europe, the northern United States, and the North Carolina mountains of the United States. CM has been used as a folk medicine since the 16th century. Eastern Europeans have used CM as a botanical medicine in the treatment of cancer, gastritis, ulcers, and tuberculosis (Huang, 2002). When CM is administered for extended periods, it has beneficial effects in the treatment of breast cancer, liver cancer, uterine cancer, and gastric cancer, as well as hypertension and diabetes (Jarosz, Skórska, Rzymowska, Kochmańska-Rdest, & Malarczyk, 1990; Kahlos, Kangas, & Hiltunen, 1986; Kahlos & Tikka, 1994). The most positive therapeutic effects of CM appear to be due to the presence of chemicals such as betulin and betulinic acid, isolated from the white portion of birch bark. Recent studies on phytochemicals in CM have identified polyphenolic compounds, triterpenoids, steroids and betulin, which have various pharmacological activities. Park, Lee, Jeon, Jung, and Kang (2004) reported that polyphenolic extracts of CM protect against oxidative damage to DNA in human lymphocytes (Park et al., 2004). Studies of the endo-polysaccharides of CM have demonstrated that they have anti-inflammatory and anti-tumor activities (Kim et al., 2005, 2006). CM is traditionally administrated as a food in the form of a hot water extract, prepared from a small piece of the mushroom or crushed mushroom and such aqueous extracts have been reported to exhibit anti-mitotic activity (Burczyk, Gawron, Slotwinska, Smietana, & Terminska, 1966). In our previous study, the antioxidant activity of phenolics (free, esterified and insoluble-bound forms) in Rubi Fructus was determined. The content of esterified or insoluble-bound phenolics is important to present the antioxidant activity, because whole fruits or grains are usually administrated (Ju et al., 2009; Liyana-Pathirana & Fereidoon, 2006). In this study, however, the released soluble phenolic acids were focused because the CM is only administrated as a hot water extract. Recently, several attempts have been made to release bound compounds via processes such as steam treatment, fermentation, and protease treatment, in attempts to enhance the pharmacological activities (Jeong et al., 2004). Choi, Lee, Chun, Lee, & Lee, 2006 showed that free polyphenolic and flavonoids are released by heat treatment more easily than raw materials due to changes in the matrix as the result of the disruption of the plant cell walls. They proposed that the enhanced radical scavenging activities are correlated with the increased free phenolics

* Corresponding authors. Tel.: +82 2 880 7844; fax: +82 2 886 7844 (S.W. Kwon), tel.: +1 214 645 6163 (J. Lee).
E-mail addresses: jeongmi.lee@utsouthwestern.edu (J. Lee), swkwon@snu.ac.kr (S.W. Kwon).

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content. Dewanto and colleagues also reported that the free phenolics levels are increased, whereas bound phenolics levels are decreased by steaming (Dewanto, Wu, & Liu, 2002). Based on these bio-transformation processes, it would be expected that a steam treatment of CM might release phenolic acids from the cell wall, thereby enhancing the antioxidant activity of CM. The aims of the present study were, therefore, to determine the phenolic compounds liberated by the steam treatment of CM and to evaluate changes in their antioxidant activity.

2. Materials and methods

2.1. Chemicals and reagents

I. obliquus (Chaga Mushroom, CM) was purchased from a local Kyoung-dong market in Seoul, Korea. Phenolic acid standards, 2,5-dihydroxyterephthalic acid, Folin–Ciocalteu's phenol reagent, 1,1-diphenyl-2-picrylhydrazyl, N,O-bis(trimethylsily)trifluoroacetamide with 1% trimethylchlorosilane, and pyridine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was acquired from Fluka. HPLC grade water and acetonitrile were obtained from Duksan (Ansan, Kyungki-Do, Korea).

2.2. Sample preparation

CMs were pulverised with an electric mill (Shinil model SFM-555SP, Hwasung, Korea) and sieved using a standard sieve (300 μm). Thirty grams of powdered CM was transferred into a Duran bottle and steam-treated at 120 °C for 3 h in an autoclave. Free phenolic acid extracts were obtained according to a previously reported method (Krygier, Sosulski, & Hogge, 1982). Briefly, the samples were extracted three times with a mixture of 70% methanol and 70% acetone (1:1) at room temperature, and centrifuged. The supernatant was evaporated to remove organic solvents and acidified (pH = 2). After centrifugation, the aqueous phase was extracted three times with a 1:1 mixture of diethyl ether and ethyl acetate (DE/EA). The organic phase was collected, and the solvent was removed under reduced pressure to give the free phenolic acids fraction.

2.3. Determination of soluble phenolic content

The soluble phenolic content in the extracts was determined using the modified Folin–Ciocalteu method, which was based on the method described by Singleton and Rossi (Singleton & Rossi, 1965). Forty microlitres of the diluted CM methanolic solution were added to 1.8 mL of 0.02 M Folin–Ciocalteu's phenol reagent solution (aq.) and the resulting solution was allowed to stand at room temperature (RT) for 5 min; 1.2 mL of 15% Na_2CO_3 was subsequently added to the mixture. After incubation at RT for 90 min, the absorbance of the solution was measured at 765 nm by microplate spectrometry (Spectramax 340PC, Molecular Devices, Sunnyvale, CA, USA). Gallic acid was used as a standard, and the soluble phenolic content was expressed as milligrams of gallic acid per 100 g of sample.

2.4. DPPH radical scavenging activity

The antioxidant activity of each fraction was tested by measuring DPPH radical scavenging activity using Blois's method with minor modifications (Blois, 1958). Each extract was dissolved in methanol and repeatedly diluted to yield a concentration range of 0.1 μg/mL–0.2 mg/mL. A mixture of 100 μL of diluted sample solution and 100 μL of a 200 μM DPPH (1,1-diphenyl-2-picrylhydrazyl) methanolic solution was incubated at RT in the dark for 30 min. Afterwards, the plate was agitated slightly for 5 min, and the absorbance measured at 517 nm.

2.5. LC/PDA and ESI LC/MS analysis of CM extracts

To analyse the phenolic acids in the untreated and steam-treated CM extracts, the extracts were dissolved in 20 mL of methanol and sonicated for 5 min. Then, 150 μL of filtered supernatant was transferred, by means of a PTFE 0.50 μm syringe filter, into glass inserts in the vials. The standard stock solution, which included four phenolic acids used for calibration, was prepared in a concentration range from 0.1 to 5 mg/mL. The components of the CM extracts were separated using a reverse-phase column (Synergi Hydro-RP, 250 × 4.60 mm, i.d., 4 micron, Phenomenex, Torrance, CA) and analysed by LC/PDA (Hewlett Packard series 1100, Agilent, USA). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 100% acetonitrile (solvent B). The non-linear gradient elution used was as follows: 5% B at 0 min, 15% B at 20 min, 30% B at 50 min, 50% B at 65 min, and 95% B at 75 min. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. Electrospray ionisation (ESI) mass spectrometry (LCQ, Finnigan Mat, San Jose, CA, USA) was used to identify phenolic acids in both the positive and negative modes. The same LC conditions of LC/PDA were applied to the ESI LC/MS analysis. The capillary temperature was 250 °C, and the capillary voltages were adjusted to +10 V for the positive mode and −10 V for the negative mode.

2.6. GC/MS analysis of CM extracts

In preparation for the GC/MS analysis, phenolic acids fractions were silylated by incubation at 60 °C for 15 min with 200 μL of pyridine and 300 μL of TMS reagent. An Agilent 6890 Series Plus gas chromatograph (Agilent Technologies, USA) equipped with a JMS-GC mate (JEOL, Japan) mass spectrometer was used for the analysis. The silylated compounds were separated on a HP-5 capillary column (60 m × 0.25 mm I.D., 0.25 μm film thickness, Hewlett Packard, USA). The column temperature was maintained at 100 °C for 2 min and then increased to 270 °C at a rate of 5 °C/min. After the temperature reached 270 °C, it was held there for 6 min. The temperature of the ion source and the transfer line was 230 °C. The inlet temperature was 250 °C, the flow rate was 1.2 mL/min, and the carrier gas was helium.

2.7. Statistical analysis

All experiments were conducted independently in triplicate, and the data were expressed as the mean ± S.D. (standard deviation). The experimental data were subjected to one-way analysis of variance (ANOVA) to compare the antioxidant activity and phenolic acid content in each sample. Differences were considered to be statistically significant if the p value was less than 0.05.

3. Results and discussion

3.1. Soluble phenolic content in CM extracts

To determine the effects of steam treatment on CM, CM samples were incubated in an autoclave (120 °C, 3 h). Differences between untreated and treated CM are normally observed at temperatures over 100 °C, and a temperature-stepwise study on water extractions showed that the total extractable phenolics were increased in volume at 120 °C (Lee, Kim, Kim, & Choi, 2005). The soluble phenolic content of the CM extracts were 58.7 mg of GAE/100 g (gallic acid equivalent per 100 g of dried CM), and this value increased to 125 mg GAE/100 g in steam-treated CM. The increased soluble

Table 1
Soluble phenolic content and DPPH radical scavenging activities of *Inonotus obliquus* extracts.

Fractions	Soluble phenolic content GAEmg/100g[a]	
	Untreated	Steam-treated
Methanol:Acetone (70:70, v/v) extract	203 ± 4.2	258 ± 5.9*
Free phenolics	58.7 ± 5.4	125 ± 5.2*
	DPPH IC_{50}(mg/mL)[b]	
	Untreated	Steam-treated
Methanol:Acetone (70:70, v/v) extract	3.2 ± 0.11	2.0 ± 0.04*
Free phenolics	21 ± 3.38	7.8 ± 0.13*
Standard mixture[c]	51 ± 0.87	9.6 ± 1.9*

Values are expressed as means ± S.D. (n = 3).
* The activities of steam-treated samples differed significantly from those of the untreated samples ($p < 0.001$).
[a] Gallic acid equivalent mg per 100 g of CM.
[b] Milligram of dry weight of CM in methanol solution.
[c] Four phenolic acids were mixed (vanillic acid, protocatechuic acid, syringic acid, and 2,5-dihydroxyterephthalic acid).

phenolic content may be due to the liberation of phenolics as the result of the disruption of cell walls during the steam treatment. However, the Folin–Ciocalteu method is not specific for quantification of phenolic acids because the reagent used in the method could generate additive reactions due to the interaction with reducing non-phenolic compounds (Ainsworth & Gillespie, 2007). To identify the released free phenolic acids by steam treatment, the extracts were analysed by LC/MS and GC/MS.

3.2. Effects of steam treatment on antioxidant activities of CM extracts

The DPPH free radical scavenging activity was expressed as IC_{50} (Table 1). The IC_{50} value of a fraction extracted with a mixture of 70% methanol and 70% acetone (1:1) in CM was decreased from 3.2 to 2.0 mg/mL after the steam treatment. The IC_{50} value of the phenolic acids obtained from this fraction was decreased from 21 to 7.8 mg/mL by the steam treatment. Cui, Kim, and Park (2005) reported that polyphenolic extracts from CM exhibited strong antioxidant activity and their results for DPPH radical scavenging activity were similar to ours in fraction of raw material.

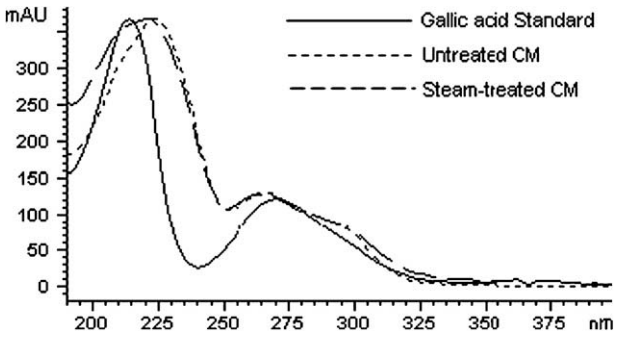

Fig. 2. PDA spectra of 2,5-dihydroxyterephatalic acid.

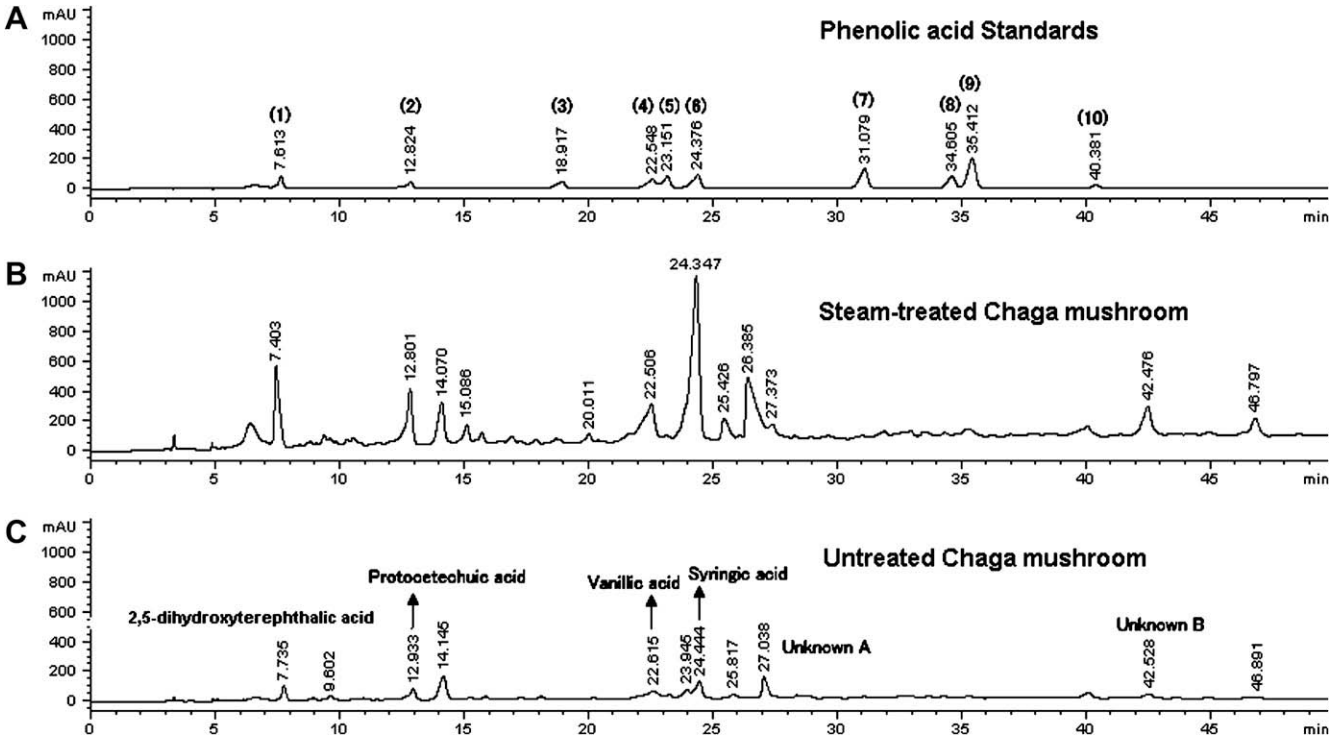

Fig. 1. LC chromatogram of phenolic acid standards and CM extracts (λ = 280 nm) (A) 10 standard mixtures: 1: Gallic acid, 2: Protocatechuic acid, 3: p-Hydroxybenzoic acid, 4: Vanillic acid, 5: Caffeic acid, 6: Syringic acid, 7: p-Coumaric acid, 8: Ferulic acid, 9: m-Coumaric acid, 10: Salicylic acid; (B) Steam-treated CM; (C) Untreated CM.

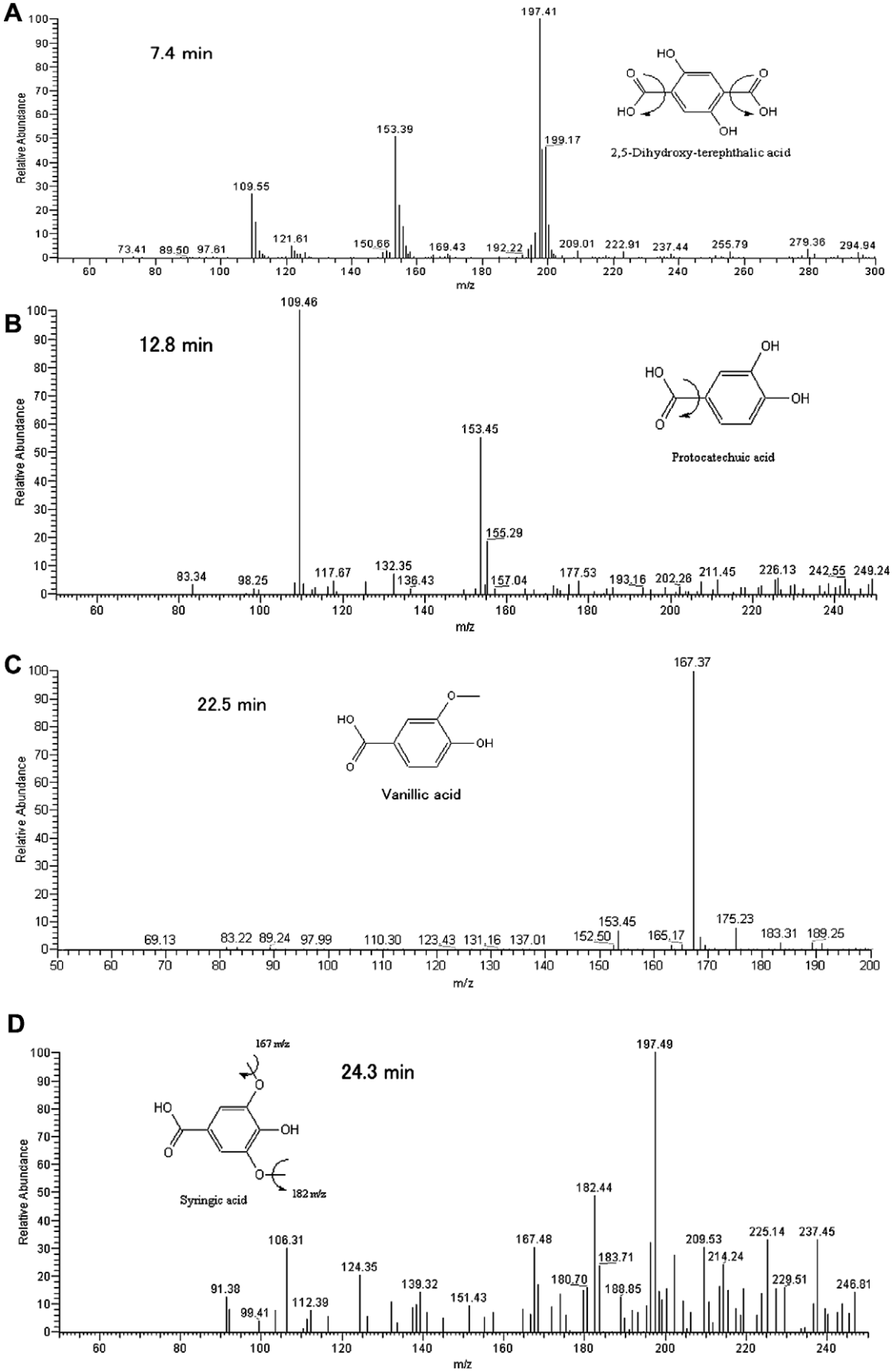

Fig. 3. Liquid chromatography mass spectra of phenolic acids. ESI LC/MS: (A) 2,5-Dihydroxyterephthalic acid; (B) Protocatechuic acid; (C) Vanillic acid; (D) Syringic acid.

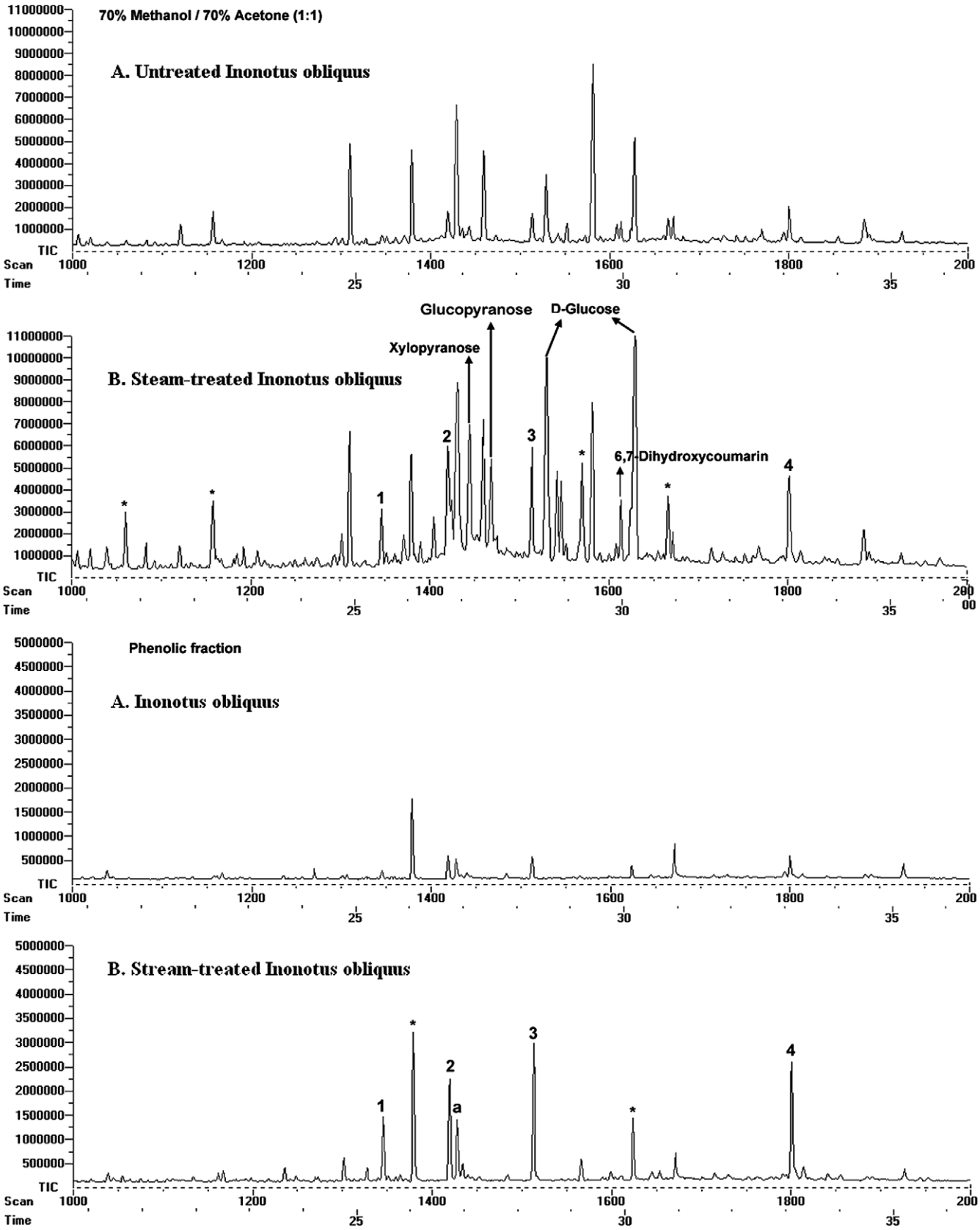

Fig. 4. GC total ion chromatograms of 70% methanol/70% acetone(1:1, v/v) fraction (up) and phenolic fraction (down): Untreated CM (A), Steam-treated CM (B); 1: Vanillic acid, 2: Protocatechuic acid, 3:Syringic acid, 4: 2,5-Dihydroxyterephthalic acid, a: Isocitric acid. Asterisks denote the presence of unknown compounds amongst the increased peaks.

Table 2
Peak identification by LC/MS and GC/MS and related DPPH scavenging activity.

Peak number	Compound	MW	LC/MS (m/z)	GC/MS (m/z)		Retention time(min)		DPPH
			$[M-H]^-$	Silylated compound		LC	GC	IC_{50} (μg/mL)[a]
1 (4[b])	Vanillic acid	168	167	312 (di-TMS)	297,223,165,73(100)	22.5	25.3	20.7 ± 1.6
2 (2[b])	Protocatechuic acid	154	153	370 (tri-TMS)	355,223,193,73(100)	12.8	26.4	4.70 ± 0.13
3 (6[b])	Syringic acid	198	197	342 (di-TMS)	327,312,297,73(100)	24.3	28.2	8.54 ± 0.55
4	2,5-dihydroxyterephthalic acid	198	197	486 (tetra-TMS)	471,383,309,73(100)	7.4	33.1	4.76 ± 0.02
a	Isocitric acid	192	191	465 (tetra-TMS)	375,273,147,73(100)	35.3[*]	26.5	7230 ± 1400

Values are expressed as means ± S.D. ($n = 3$).
[*] Retention time in ESI LC/MS.
[a] Microgram of phenolic standard in methanol solution.
[b] Peak number on LC chromatogram (Fig. 1).

3.3. Identification of phenolic acids in CM extracts

The phenolic acids in CM were analysed by LC/PDA (Fig. 1). Protocatechuic acid, vanillic acid, and syringic acid were identified by retention time and PDA spectral pattern comparison with standards ($\lambda = 280$ nm). However, a peak with a retention time at around 7.4 min was not identified by LC/PDA, since the pattern of the PDA spectrum was different from that of gallic acid, which had a similar retention time (ca 7.6 min) (Fig. 2). Through ESI LC/MS and GC/MS analyses, the peak was determined to be 2,5-dihydroxyterephthalic acid (197 m/z $[M-H]^-$) (Fig. 3A). ESI mass spectra of protocatechuic acid, vanillic acid, and syringic acid are also shown in Fig. 3. The LC chromatograms shown in Fig. 1, therefore, revealed that the levels of the four identified phenolic acids were significantly increased as the result of the steam treatment. The GC–MS total ion chromatograms of the solvent mixture (70% methanol:70% acetone = 1:1) fraction and the phenolic fraction in CM are shown in Fig. 4. Vanillic acid, protocatechuic acid, syringic acid, and 2,5-dihydroxyterephthalic acid were also identified by GC/MS, as they were by LC/MS, and their amounts were also increased by the steam treatment. Some of the identified compounds, which were confirmed by retention time and mass spectral comparison with standards in this study, have also been identified by NMR and HR–ESI–MS in a previous report (Nakajima, Sato, & Konishi, 2007). D-glucose, glucofuranose, xylopyranose, and 6,7-dihydroxycoumarin were also increased in the fraction obtained by solvent mixture extraction after the steam treatment (Fig. 4). D-glucose was identified by comparison of its GC retention time and mass spectrum with a standard, and glucofuranose, xylopyranose, and 6,7-dihydroxycoumarin were confirmed by a GC/MS library search (Nist search). Glucose, in conjunction with amino acids, provides an enhanced antioxidant activity because of its participation in the Maillard reaction, but the increased level of glucose suggests that it does not react with other compounds and thereby does not contribute to radical scavenging activity (Kim & Lee, 2009). The level of its antioxidant activity was low and other compounds, glucofuranose and xylopyranose, are estimated to be low due to similar structure. Therefore, we focused on the increased phenolic acids, compounds that are well known to possess antioxidant activity (Table 2). Individual IC_{50} values of the four increased phenolic acids (peak numbers 1, 2, 3, and 4 in Fig. 4) in the phenolic fraction are summarized in Table 2. Although some peaks in Fig. 4 were not identified, it is likely that the greatest proportion of antioxidant activity was due to the four identified phenolic acids. To evaluate the antioxidant capacity contributed by the four phenolic acids in untreated and steamed CM, the peaks were quantified by HPLC/PDA. The amounts of the four phenolic acids (Fig. 1C) in untreated CM were 0.19, 0.23, 0.22, and 0.29 mg/mL, and the amounts for each peak in the steamed CM were increased to 0.76, 0.90, 1.44, and 1.90 mg/mL, respectively. The results of the DPPH test, performed using the standard mixture which included the quantified amount of the four phenolic acids, showed that the four phenolic acids contributed 41% of the total antioxidant activity in untreated CM, and 81% of the total antioxidant activity in the steamed CM (Table 1). The contribution ratio out of total antioxidant activity (%) was calculated based on the following equation; [contribution ratio = ($1/IC_{50}$ value of four phenolic acids)/($1/IC_{50}$ value of untreated or steam-treated CM)/X 100]. Therefore, it can be concluded that the increased phenolic contents after the steam treatment contributed to the decreased DPPH IC_{50} value and the fortified antioxidant activity (Table 1). Caffeic acid identified by GC/MS (tri-TMS; 396 m/z) was also found in both fractions, but only trace amounts were present. Isocitric acid was identified in the chromatogram of the phenolic fraction and its presence confirmed by GC/MS. This compound may have been produced from a fatty acid during the fatty acid elimination procedure. However, isocitric acid had no activity as a radical scavenger although its levels were increased in the phenolic fraction of the steamed CM. The IC_{50} value of isocitric acid was also estimated (Table 2).

4. Conclusions

The soluble phenolic content and DPPH radical scavenging activity of extracts obtained through the steam treatment of Chaga mushroom (CM) were measured. The amounts of soluble phenolic acids of the CM and the antioxidant activity, which were estimated by DPPH radical scavenging, were enhanced as the result of the thermal processing. The volume-enhanced compounds, identified by LC/PDA, ESI LC/MS, and GC/MS, were determined to be vanillic acid, protocatechuic acid, syringic acid, and 2,5-dihydroxyterephthalic acid. These results show it is possible to increase the amounts of free phenolic compounds liberated by the disruption of cell walls by steaming and that the enhanced radical scavenging activity is due to the release of increased amounts of phenolics, which directly affect radical scavenging activity.

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