Extraction and Identification of Antioxidants in the Roots of Yacon (*Smallanthus sonchifolius*)

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Yacon, *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson, Asteraceae, an important economic species grown for its juicy tuberous root, is potentially beneficial in the diet to diabetics. The antioxidative activity of yacon root was studied by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Antioxidants were extracted by methanol and isolated and purified by gel permeation chromatography and preparative reverse-phase HPLC. Two of the major antioxidants were identified as chlorogenic acid and tryptophan by NMR and mass spectrometry.

Keywords: *Yacon; Smallanthus sonchifolius; antioxidant; chlorogenic acid; tryptophan; Asteraceae*

INTRODUCTION

Yacon, *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson, has long been an important economic species grown for its juicy tuberous root in south America since ancient times, on the eastern slopes of the Andes from Venezuela to northwestern Argentina (Zardini, 1991). Yacon has abundant fructans but lacks starch, which makes it potentially beneficial in the diet of diabetics (Nakanishi, 1997). It has been cultivated in several trial plant fields in northern Japan and may become commercially available in the foreseeable future.

Yacon belongs to Asteraceae, a family featuring plants such as burdock (*Arctium lappa*) and garland (*Chrysanthemum coronarium*), which contain considerable amounts of phenolic compounds showing antioxidative activity (Maruta et al., 1995; Chuda et al., 1998). As natural dietary antioxidants, phenolic compounds from plants may protect cell membranes against damage by oxygen radicals. Therefore, investigation of such antioxidative phenolic compounds is very important to elevate its dietary value and expand its cultivation and market potential. It has been found that yacon contains a considerable amount of phenolic compounds, as much as 3.8% on a dry weight basis (Folin-Denis method, chlorogenic acid as standard, personal data). In the present study, we determined the antioxidative activty of yacon root by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, isolating and characterizing antioxidative principles.

MATERIALS AND METHODS

Materials. Fresh roots of the yacon were harvested at Shikoku Agricultural Experimental Station and stored at 4 °C until use.

Chemicals. *d*-Dimethyl sulfoxide (DMSO) and *d*-methanol were obtained from E. Merck (Darmstadt, Germany). DPPH, β-carotene, 2-deoxy-D-ribose, chlorogenic acid (CA), and L-tryptophan were obtained from Wako (Osaka, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylic acid) was obtained from Aldrich (Milwaukee, WI). Tween 20 (polyoxyethylene sorbitan monopalmitate) and linoleic acid (*cis,cis*-9,12-octadecadienoic acid) were obtained from Nacalai Tesque (Kyoto, Japan).

Antioxidative Activity Screening. DPPH assay was modified from Brand-Williams (1995) as follows: DPPH solution was prepared at a concentration of 2×10^{-4} mol/L in methanol. During the test, 2 mL of an organic solvent extract was mixed with 2 mL of DPPH solution. The test tube was capped after nitrogen bubbling and placed in the dark. After standing for 60 min at 20 °C, absorbance was measured at 517 nm by a UV−vis spectrophotometer (UV-1200, Shimadzu Co, Japan). All samples were made in triplicate, with Trolox as a positive control.

Extraction and Fractionation of Crude Extract. Some 1.4 kg of fresh yacon was peeled and cut into small pieces (0.2 cm), and extracted with 1.5 L of methanol overnight at 4 °C. The extract solution was concentrated to dryness in vacuo and dissolved in 40 mL of 10% methanol in water. Then the solution underwent gel permeation chromatograph (GPC) on a 450 × 40 mm column (Toyopearl HW-40, TOSOH, Tokyo, Japan). It was first washed with 200 mL of distilled water to remove viscous carbohydrates and then eluted at a linear gradient mode from 5% to 90% of methanol in water containing 5% acetic acid. Each fraction was 15 mL, and the total number of eluted fractions was 120. Eluted fractions were analyzed by DPPH assay as stated above. It was observed that most of the tubes showed antioxidative activity. Since the initial fraction (tubes 1−25) contained rather large amounts of carbohydrates, which could interfere with subsequent isolation, this fraction was abandoned and the remaining active fractions (tubes 26−50) were combined and subjected to preparative isolation and purification.

Isolation of Active Compounds by Preparative HPLC. The preparative HPLC system consisted of an 880-PU pump (Jasco, Tokyo, Japan), a manual injector (Model 7125, Rheodyne, Cotati, CA) with a 5.0 mL sample loop, and an 875-UV variable wavelength detector. The detector was set at 280 nm. The preparative column was a Wakopak C18 reversed-phase column (Wakosil-II 5C18 HG prep, 250 × 20 mm, Osaka, Japan). The mobile phase was in isocratic mode, as 8% methanol in 5% aqueous acetic acetic acid, with a flow rate of 8 mL/min. Two compounds were isolated from this chromato-

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Table 1. DPPH Scavenging Activity of Yacon Extracts

sample	weight (g)	solvent (mL)	inhibiton (%) mean ± SD ($n = 3$)
lyophilized yacon	0.5	$CHCl_3$, 10	13.8 ± 4.7
lyophilized yacon	0.5	EtOAc, 10	17.0 ± 5.5
lyophilized yacon	0.5	Me_2CO, 10	20.5 ± 6.4
lyophilized yacon	0.5	MeOH, 10	93.2 ± 0.6
Trolox	0.0025	MeOH, 100	85.5 ± 0.5

gram and designated as compound A (retention time 24.1 min) and compound B (retention time 26.6 min). After lyophilization, compound A appeared as a colorless powder, weighing 3.7 mg; the UV spectrum showed a 280 nm absorption. Compound B appeared as a light green powder, weighing 5.8 mg; the UV spectrum showed a 331 nm absorption, with a shoulder peak at 308 nm. (Only part of the collected eluent was used in this section because both compounds were identified with a known structure.)

Evaluation of Antioxidant Activity by β-Carotene and Deoxyribose Method. Deoxyribose assay was according to Aruoma (1994). Antidiscoloration of β-carotene was according to Chuda et al. (1996).

Instrumental Analysis. Mass spectra were obtained on a SX-102 spectrometer (JEOL, Akishima, Japan) by fast atom bombardment (FAB) ionization in positive mode. NMR data for ^1H at 300.13 MHz and ^{13}C at 75.48 MHz were recorded on a DRX-300 spectrometer (Bruker, Karlsruhe, Germany) at room temperature. Chemical shifts were reported relative to tetramethylsilane as an internal standard.

Preliminary Quantification of Chlorogenic Acid and Tryptophan in Yacon. The content of chlorogenic acid and tryptophan in yacon was determined by HPLC analysis, conducted using a Jasco PU-980 HPLC pump (Tokyo, Japan) equipped with a Jasco-JMBS multiple wavelength photodiodide detector with a DP-L910W Borwin-PDA program. The column (150 × 6 mm i.d., Cosmosil 5 C18-MS, Nacalai Tesque) was placed in a Jasco CO-965 thermostated control system, operated at 40 °C throughout analysis. The mobile phase consisted of isocratic 5% methanol in 5% aquesous acetic acid, operating at a flow rate of 1.0 mL/min, injection volume 10 μL.

Fresh yacon sample (ca. 2.0 g) from six different tubers was extracted by 15 mL of 5% methanol in 5% aquesous acetic acid, after sonication for 15 min twice, overnight. The extract was filtrated through a 0.45 μm membrane, and the filtrate was adjusted to a volume of 20 mL with the above solvent. Chlorogenic acid and tryptophan in the extract were identified by the retention time and a spiking experiment with authentic compounds. Quantification was calculated from the calibration curve of authentic chlorogenic acid and tryptophan.

RESULTS AND DISCUSSION

DPPH Scavenging Activity of Yacon Extracts. As expected, yacon extracts showed obvious DPPH scavenging activity. Table 1 indicated that extracts by chloroform, ethyl acetate, and acetone had similar activities, while methanol extract had a much higher activity than the other three less polar solvents. Methanol was, therefore, chosen for scale-up extraction for antioxidant isolation and identification.

Identification of Compounds A and B. The ^1H NMR spectrum of compound A showed that four protons with chemical shifts at 6.96, 7.05, 7.33, and 7.55 ppm belonged to vicinal aromatic hydrogens, deduced from their coupling pattern and cross-peaks in a H−H COSY experiment. The ^{13}C NMR spectrum and DEPT experiment showed this compound to have 4 *tert*-carbons, 6 CH, and only one CH_2. The FAB-MS spectrum of compound A was as follows: *m/z* 205 ($[M + H]^+$, relative intensity 100%), 227 ($[M + Na]^+$, 18), 297 ($[M + H +$ glycerol$]^+$, 46), and 319 ($[M + Na +$ glycerol$]^+$, 11). It contained a fragment peak having *m/z* 130 and a relative intensity of 53. Combined with carbon chemical shift data, it appeared to be indole−methylene skeleton. So we assigned this compound as tryptophan, molecular weight 204; the same as compound A. Detailed ^1H and ^{13}C NMR assignments are shown in Table 2.

Table 2. ^1H NMR and ^{13}C NMR Spectral Data of L-Tryptophan

	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
	ppm	*J*, Hz	ppm	*J*, Hz	ppm	DEPT
1					138.41	C
2					128.48	C
3	7.55, d	8.1	7.69, d	7.8	122.75	CH
4	7.05, td	7.6, 6.3	7.11, td	7.9, 1.1	120.12	CH
5	6.96, td	7.2, 7.2	7.04, td	7.9, 1.1	119.34	CH
6	7.33, d	7.9	7.35, d	7.9	112.43	CH
7					109.57	C
8	7.17, s		7.18, s		125.13	CH
9			3.14, 2dd	9.4, 5.8	28.46	CH_2
9			3.51, 2dd	15.3, 4.5		
10			3.85, dd	9.4, 4.0	56.73	CH
11					174.44	C

Compound B was presumed to be chlorogenic acid or its derivative, based on its UV spectrum. The FAB-MS spectrum of compound B was as follows: *m/z* 355 ($[M + H]^+$, relative intensity 22%), 377 ($[M + Na]^+$, 57), and 399 ($[M + 2Na - H]^+$, 11). Its molecular weight, 354, corresponds to chlorogenic acid. ^1H NMR spectrometry confirmed this assumption to be correct. Detailed ^1H NMR assignment is as follows: H-2, 2H, 2.07 ppm, m; H-6, 2H, 2.19 ppm, m; H-4, 1H, 3.72 ppm, dd; H-5, 1H, 4.16 ppm, ddd; H-3, 1H, 5.32 ppm, ddd; H-8′, 1H, 6.25 ppm, d; H-5′, 6.76 ppm, d; H-6′, 694 ppm, dd; H-2′, 7.04 ppm, d; H-7′, 7.55 ppm, d. This compound was assigned as chlorogenic acid.

Compared with authentic L-tryptophan and chlorogenic acid, they have the same retention times on HPLC and almost identical ^1H NMR spectra.

Antioxidative Activity of L-Tryptophan and Chlorogenic Acid. The two identified components, namely chlorogenic acid and L-tryptophan are well-studied antioxidants. Chlorogenic acid and its derivatives are typical antioxidants in terrestrial plants including the potato, apple, and tomato (e.g., Ohnishi et al., 1994). L-Tryptophan is an essential amino acid for animals and human beings, also known as a precursor of melatonin and serotonin. Its antioxidative activity has recently received much attention. Konturek et al. (1997) observed that melatonin and L-tryptophan protected the gastric mucosa from oxidative damage such as stress and ischaemia; Duell et al. (1998) demonstrated that L-trytophan is more potent than melatonin in scavenging radicals either from metal-ion-mediated or macrophage-mediated oxidation of low-density lipoprotein (LDL). In evaluating their antioxidative activity (Figure 2), we found that L-tryptophan has only a very slight DPPH scavenging activity while chlorogenic acid was very active. In DPPH assay, L-tryptophan was only found because of its coexistence in the chlorogenic acid fraction. A brief evaluation of antioxidative activity by β-carotene and deoxyribose assays showed that both L-tryptophan and chlorogenic acid protect β-carotene from discoloration. Deoxyribose assay showed that only L-tryptophan scavenged hydroxyl radicals and chlorogenic acid stimulated iron-mediated hydroxyl radical production to some extent (Table 3). The antioxidative mechanism between chlorogenic acid and L-tryptophan thus appears to be different toward radical species.

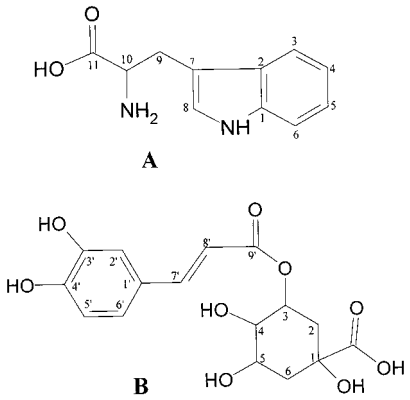

Figure 1. Structures of tryptophan (A) and chlorogenic acid (B).

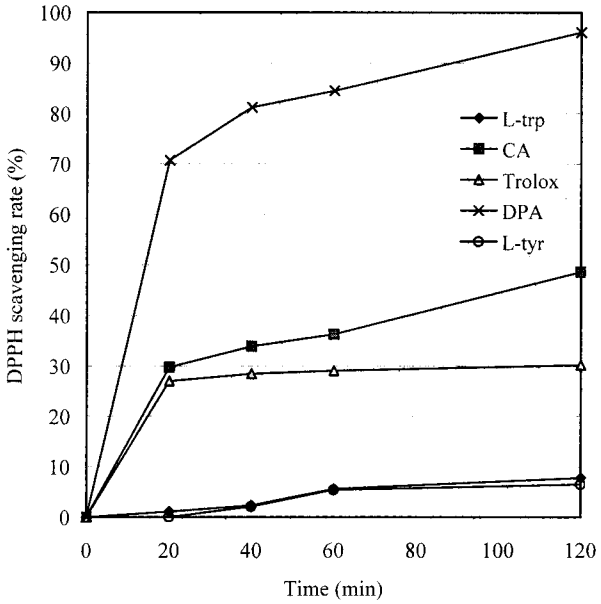

Figure 2. DPPH scavenging activity of antioxidants. The concentration of the DPPH solution and each tested compound, namely, L-tryptophan (L-trp), chlorogenic acid (CA), Trolox, 3,4-dihydroxyphenylacetic acid (DPA), and L-tyrosine (L-tyr), was 2×10^{-4} mol/L; 3.5 mL of DPPH solution was mixed with 0.5 mL of the tested compound solution, and the change of absorbance was measured at 517 nm.

Table 3. Deoxyribose Assay and β-Carotene Assay for L-Tryptophan and Chlorogenic Acid[a]

	deoxyribose assay (%)	β-carotene assay (%)
positive control	35.5 ± 4.2	100
L-tryptophan	57.5 ± 3.4	55.4 ± 6.7
chlorogenic acid	−5.5 ± 2.7	75.5 ± 4.2

[a] Positive control was Trolox in both cases. In the deoxyribose assay, compound concentration was 20 mmol/L in the final solution. In the β-carotene assay, trolox antidiscoloration was considered 100% and the compound concentration was 10 ppm in the final solution.

Preliminary Quantification on Chlorogenic Acid and Tryptophan in Yacon. Figure 3 shows an HPLC chromatogram of freshly prepared authentic chlorogenic acid and tryptophan (each with 0.05 mg/mL). The retention time (R_t) of L-tryptophan and chlorogenic acid was 5.65 and 6.81 min, respectively. By calculating from the authentic standard curve, the contents of chlorogenic acid in yacon was 48.5 ± 12.9 μg/g and tryptophan, 14.6 ± 7.1 μg/g (fresh weight), determined from six different yacon tubers.

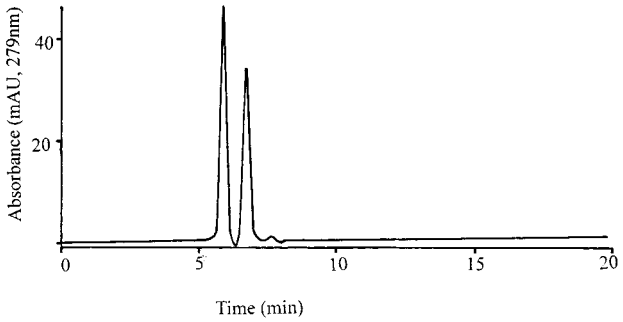

Figure 3. HPLC chromatogram of freshly prepared authentic chlorogenic acid and tryptophan (each with 0.05 mg/mL). Peformed by a Jasco PU-980 HPLC pump (Tokyo, Japan), a Jasco-JMBS multiple wavelength photodiodide detector with a DP-L910W Borwin-PDA program. The column (150 × 6 mm i.d., Cosmosil 5 C18-MS, Nacalai Tesque) was in a Jasco CO-965 thermostated oven at 40 °C. The mobile phase was isocratic 5% methanol in 5% aquesous acetic acid, operating at a flow rate of 1.0 mL/min, sample injection volume is 10 μL. The retention time (R_t) of L-tryptophan and chlorogenic acid was 5.85 and 6.81 min, respectively.

L-Tryptophan had not previously been known as a plant antioxidant, and more detailed quantification of L-tryptophan and chlorogenic acid upon cultivar, plant part, growth cycle, and cooking loss in yacon is still needed.

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