Food Sci. Biotechnol. Vol. 15, No. 2, pp. 220 ~ 226 (2006)

Food Science and Biotechnology

Separation of Lipid-Soluble Component to Decrease Thermal Oxidation of Lard from Spinach (*Spinacia oleracea*)

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Abstract Component having antioxidant activity on lard during heating was separated from hexane extract of spinach, and its characteristic chemical structure was speculated through nuclear magnetic resonance spectroscopy, liquid chromatography-mass spectrometry, and Fourier transform infrared spectrophotometry. Lard was heated with hexane-, ethyl ether-, ethyl acetate-, or ethanol extract of spinach at 180°C for 20 hr. Hexane extract of spinach, having highest antioxidant activity on lard during heating, was fractionated by silicic acid column chromatography (SACC), and SACC fractions having higher antioxidant activity on lard during heating were further separated by thin layer chromatography (TLC). Isolated compound from SACC fractions of hexane extract of spinach by TLC had sugar moieties and benzene ring along with hydroxy, carbonyl, and alkyl groups in the structure.

Key words: hexane extract of spinach, antioxidant, heating, lard, structure

Introduction

Antioxidants are widely used in lipids and lipid-containing foods to slow down oxidation. Extracts of vegetables, tea, and herbs have been reported to show antioxidant activities in lipids (1-3), among which hexane extract of burdock (3) and ethanol extract of oregano (4, 5) significantly reduced thermal oxidation and autoxidation of lard, respectively. In addition, methanol extract of tea was effective in the autoxidation and thermal oxidation of rapeseed oil (6).

Spinach (*Spinacia oleracea*) was reported to decrease the oxidation of lipid in foods (7, 8). Spinach powder added to dough improved the oxidative stability of fried dough during storage (9). In addition, water extract of spinach showed antioxidant activity by scavenging linoleic acid radicals (8) and superoxide anion radicals (10). Flavonoids and *p*-coumaric acid derivatives, in particular spinacetin and paluletin, were major compounds responsible for the antioxidant activity of water-soluble extract of spinach (11). However, water-soluble extract of spinach itself is not useful in preventing lipid oxidation due to its solubility restriction. Unfortunately, no study has yet been performed on the effects of lipid-soluble extract of spinach on lipid foods. This study was performed to investigate the plausibility of lipid-soluble extract of spinach as a natural-grade antioxidant in lipid foods by determining the antioxidant activities of organic solvent extracts of spinach on lard during heating and determine the chemical structure of a compound responsible for the antioxidative activity.

Materials and Methods

Materials and chemicals Lard was provided by Heinz Co. Ltd. (Seoul, Korea). Fresh spinach was purchased at a local supermarket in Incheon, washed with distilled water, and drained. It was then freeze-dried at -54°C for 16 hr and ground into 60 mesh.

Isooctane and *p*-anisidine were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan) and Fluka Chemical AG (CH, Switzerland), respectively. *n*-Hexane and precoated silica gel plate (Kieselgel 60 F_{254}) were purchased from Merck Co. (Darmstadt, Germany). Silicic acid (200-400 mesh), 14% BF_3-methanol solution, deuterated chloroform, and trifluoroacetic acid were products of Sigma-Aldrich Co. (Steinheim, Germany). Acetonitrile was purchased from Fischer Scientific Co. (Fairlawn, NJ, USA). All other chemicals were of reagent grade.

Preparation of organic solvent extracts of spinach Organic solvent extracts of spinach were prepared as shown in Fig. 1. Spinach powder (40 g) was mixed with *n*-hexane (150 mL) in a 250-mL round bottomed flask, placed in a 40°C water bath for 3 hr with an air condenser, and filtered through a Büchner funnel and Whatman filter paper (No. 42; Whatman International Ltd., Kent, England) to afford hexane extract of spinach. The residue was serially re-extracted with diethyl ether, ethyl acetate, and ethanol. Extraction conditions and methods were the same as those for hexane extract of spinach. The solvents in the filtrates were completely removed using a rotary evaporator (N-N; Rikakikai Co., Ltd., Tokyo, Japan) at 40°C, and each extract was placed in a glass bottle covered with an aluminum foil. After nitrogen flushing, the bottle was tightly sealed with an aluminum cap and a teflon-coated septum, and placed in a -40°C freezer until use.

Preparation of SACC fractions of hexane extracts of spinach Hexane extract of spinach, showing the highest antioxidative property during heating of lard, was further fractionated by silicic acid column chromatography (SACC). The column (3×30 cm) was filled with activated

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Received October 21, 2005; accepted January 25, 2006

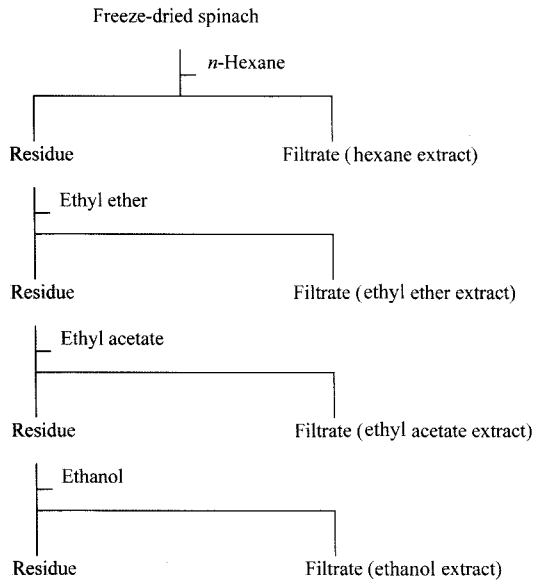

Fig. 1. Preparation of spinach extracts.

silicic acid, and the eluting solvents were mixtures of hexane and benzene at volume ratios of 100:0, 95:5, 90:10, 80:20, 50:50, and 0:100, respectively. Solvent in each fraction was evaporated to dryness using a rotary evaporator (N-N; Rikakikai Co., Ltd.) at 40°C.

Thermal oxidation of lard Lard was melted in an 80°C water bath and mixed with hexane-, diethyl ether-, ethyl acetate-, or ethanol extract of spinach. Concentrations of spinach extracts in lard were 1000 and 3000 ppm. Each SACC fraction of hexane extracts of spinach was separately added to lard at 1000 ppm. Blank sample was lard without spinach extracts. All samples were prepared in four replicates. A sample (50 g) added with each solvent extract of spinach or SACC fraction of hexane extract was placed into a 100-mL Erlenmeyer flask covered with an aluminum foil to exclude light and heated on a hot plate (Dongyang Science Co., Siheung, Korea) at 180°C for 20 hr. Samples were taken at the 10^{th} and 20^{th} hr for analyses.

Determination of lard oxidation during heating Oxidation of lard during heating at 180°C for 20 hr was monitored by determining free fatty acid (FFA) value, conjugated dienoic acid (CDA) content, and *p*-anisidine value (PAV) by AOCS methods Cd 3a-63, Ti 1a-64, and Cd 18-90 (12), respectively, and fatty acid composition. After esterification with 14% BF_3-methanol solution, the fatty acid composition was analyzed by gas chromatography (GC) using a Younglin M600L gas chromatograph (Younglin Co., Seoul, Korea) equipped with a Supelcowax capillary column (30 m × 0.53 mm, 1.0 μm thick; Supelco Inc., Bellefonte, PA, USA) and a flame ionization detector (13). Temperatures of the oven, the injector, and the detector were 210, 280, and 280°C, respectively. The nitrogen flow rate was 5 mL/min, and the split ratio was 33:1.

Separation of lard-antioxidative compounds present in hexane extract of spinach Fraction 3 and 4 of the SACC fractions of hexane-extracts of spinach were dissolved in hexane, loaded onto the precoated silica gel plate, and developed with a solvent of chloroform-ethyl acetate-methanol (1:2:15, v/v/v). The band in the plate was scraped, dissolved in chloroform-ethyl acetate-methanol (1:2:15, v/v/v), and filtered through a PTFE syringe filter (0.2 μm × 13 mm; National Scientific Co., Lawrenceville, NJ, USA). The filtrate was completely dried by nitrogen blowing.

Characterization of a chemical structure of an antioxidative component Structure of an antioxidative component separated and isolated from the hexane extract of spinach by SACC and thin layer chromatography (TLC) was studied through a combination of nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass (LC-MS) spectrometry, and Fourier transform infrared (FT-IR) spectrophotometry. NMR instrument was a Varian unity INOVA 400 (Varian Inc., Varian) and the component was dissolved in deuterated chloroform. LC-MS was performed after the compound was dissolved in *n*-hexane with Agilent 1100 series LC/MS spectrometer (Palo Alto, CA, USA) at an atmospheric pressure ionization electrospray mode. The solvent was acetonitrile containing 0.1% trifluoroacetic acid and water (50:50, v/v). FT-IR spectrum was obtained using KBr window in a Spectrum 2000 explorer (Perkin Elmer, Norwalk, CT, USA)

Statistical analysis One-way analysis of variance at 5% significance level (14) was used to analyze the oxidation data of lard.

Results and Discussion

Effects of organic solvent extracts of spinach on the oxidation of lard during heating Yields of *n*-hexane, ethyl ether, ethyl acetate, and ethanol extracts of spinach were 2.8, 1.1, 0.7, and 9.6%, respectively. Fatty acid compositions of lard added with organic solvent extracts of spinach at 1000 and 3000 ppm during heating at 180°C are shown in Table 1 and 2, respectively. Lard consisted of myristic (1%), palmitic (25-27%), palmitoleic (2-3%), stearic (12-13%), oleic (45-46%), and linoleic (12%) acids. There was no significant difference in fatty acid composition between lards added with and without spinach extracts before heating, which indicates that the organic solvent extracts of spinach did not affect fatty acid composition of lard. During heating, relative content of linoleic acid in lard decreased and that of palmitic acid increased, and thus the content ratio of palmitic acid to linoleic acid (P/L ratio) increased. This is a common phenomenon found in the oil during heating (15). The rates of P/L ratio increase with heating time in lard added with hexane extract of spinach were lower than those of lard without spinach extracts or with ethyl ether-, ethyl acetate-, and ethanol extracts. During heating no significant differences were observed in the rates of P/L ratio increase of lard added with ethyl ether-, ethyl acetate-, and ethanol extracts of spinach and that without spinach extracts. This result clearly shows the addition of hexane

Table 1. Fatty acid compositions of 1000 ppm spinach extract-added lard during heating at 180°C for 20 hr

Additive	Heating time (hr)	Relative content (%)						P/L ratio
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	
None	0[1)]	1.32±0.05	25.47±0.38	2.51±0.07	12.05±0.38	46.23±0.52	12.43±0.14	2.05
	10	1.40±0.07	26.56±0.47	2.50±0.21	12.21±0.21	46.67±0.26	10.67±0.23	2.49
	20	1.37±0.05	27.84±0.47	2.37±0.02	13.95±0.44	46.01±0.38	8.45±0.22	3.29
n-Hexane extract	0[1)]	1.35±0.12	25.96±0.59	2.41±0.22	12.63±0.24	45.24±0.66	12.42±0.26	2.09
	10	1.41±0.05	26.43±0.51	2.52±0.03	12.78±0.11	45.11±0.51	11.76±0.11	2.25
	20	1.40±0.07	27.43±0.19	2.46±0.08	13.14±0.23	45.49±0.42	10.59±0.27	2.59
Ethyl ether extract	0[1)]	1.36±0.04	26.01±0.22	2.51±0.05	12.82±0.12	45.21±0.20	12.10±0.06	2.15
	10	1.46±0.06	27.93±0.38	2.53±0.02	13.36±0.17	45.11±0.28	9.62±0.10	2.91
	20	1.56±0.06	29.36±0.40	2.59±0.03	13.90±0.18	44.82±0.23	7.78±0.26	3.78
Ethyl acetate extract	0[1)]	1.41±0.07	26.31±0.17	2.60±0.04	12.85±0.12	44.67±0.26	12.17±0.12	2.16
	10	1.42±0.06	26.66±0.17	2.55±0.04	13.57±0.11	44.96±0.14	9.85±0.12	2.81
	20	1.56±0.03	29.02±0.52	2.67±0.03	14.08±0.09	44.53±0.23	8.16±0.44	3.57
Ethanol extract	0[1)]	1.39±0.05	26.02±0.22	2.66±0.04	12.04±0.20	45.78±0.33	12.12±0.11	2.15
	10	1.48±0.02	27.51±0.30	2.72±0.08	12.56±0.23	45.56±0.19	10.17±0.35	2.71
	20	1.54±0.02	28.89±0.30	2.78±0.03	13.14±0.14	45.70±0.32	7.96±0.40	3.64

[1)]A sample at zero heating time means one analyzed immediately after temperature of the lard reached at 180°C.

Table 2. Fatty acid compositions of 3000 ppm spinach extract-added lard during heating at 180°C for 20 hr

Additive	Heating time (hr)	Relative content (%)						P/L ratio
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	
None	0[1)]	1.32±0.05	25.47±0.38	2.51±0.07	12.05±0.38	46.23±0.52	12.43±0.14	2.05
	10	1.40±0.07	26.56±0.47	2.50±0.21	12.21±0.21	46.67±0.26	10.67±0.23	2.49
	20	1.37±0.05	27.84±0.47	2.37±0.02	13.95±0.44	46.01±0.38	8.45±0.22	3.29
n-Hexane extract	0[1)]	1.40±0.08	26.07±0.09	2.51±0.10	12.62±0.15	44.98±0.45	12.42±0.28	2.10
	10	1.50±0.05	26.80±0.27	2.60±0.04	12.68±0.12	44.57±0.20	11.85±0.06	2.26
	20	1.42±0.04	26.69±0.36	2.52±0.05	13.10±0.20	45.09±0.31	11.20±0.12	2.38
Ethyl ether extract	0[1)]	1.37±0.03	25.95±0.25	2.59±0.03	12.60±0.07	45.14±0.19	12.36±0.08	2.10
	10	1.45±0.04	27.28±0.15	2.59±0.03	13.29±0.06	45.06±0.07	10.36±0.22	2.64
	20	1.48±0.04	28.30±0.29	2.58±0.02	13.89±0.09	45.24±0.18	8.52±0.24	3.32
Ethyl acetate extract	0[1)]	1.39±0.05	26.13±0.33	2.62±0.06	12.66±0.24	45.07±0.40	12.14±0.12	2.15
	10	1.35±0.03	27.42±0.25	2.51±0.07	13.33±0.12	45.54±0.24	9.87±0.33	2.78
	20	1.55±0.03	29.24±0.39	2.64±0.05	14.09±0.30	44.69±0.40	7.79±0.26	3.76
Ethanol extract	0[1)]	1.40±0.02	26.18±0.30	2.69±0.04	11.96±0.26	45.61±0.39	12.17±0.19	2.15
	10	1.40±0.02	26.86±0.27	2.66±0.03	12.53±0.11	45.40±0.28	11.15±0.23	2.41
	20	1.50±0.02	28.13±0.26	2.76±0.01	12.61±0.14	45.69±0.06	9.13±0.64	3.10

[1)]A sample at zero heating time means one analyzed immediately after temperature of the lard reached at 180°C

extract of spinach decreased fatty acid composition changes in lard during heating.

FFA values of lard added with organic solvent extracts of spinach during heating at 180°C for 20 hr are shown in Fig. 2. FFA values of lard added with organic solvent extracts of spinach were higher than that of lard without spinach extract before heating. A previous study showed that higher amount of FFA was produced in the lipid of dough added with spinach powder during frying (16). As heating time increased from 0 to 20 hr, FFA values of lard without any spinach extracts increased from 0.02 to 0.32% due to the hydrolysis of lard (9). Addition of hexane extract of spinach lowered the FFA values of lard during heating, and the values of lard with 3000 ppm extract were lower than those of lard with 1000 ppm extract. Ethyl ether, ethyl acetate, and ethanol extracts of spinach added to lard produced higher amounts of FFA during heating than the lard without spinach extracts, and higher amounts

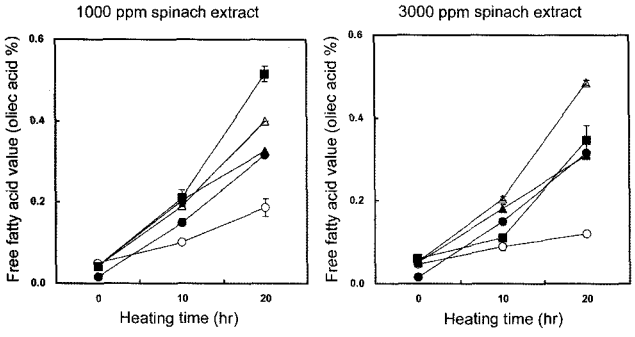

Fig. 2. Free fatty acid values of spinach extract-added lard during heating at 180°C for 20 hr. ●, No additives in lard; ○, *n*-Hexane extract; ▲, Ethyl ether extract; △, Ethyl acetate extract; ■, Ethanol extract.

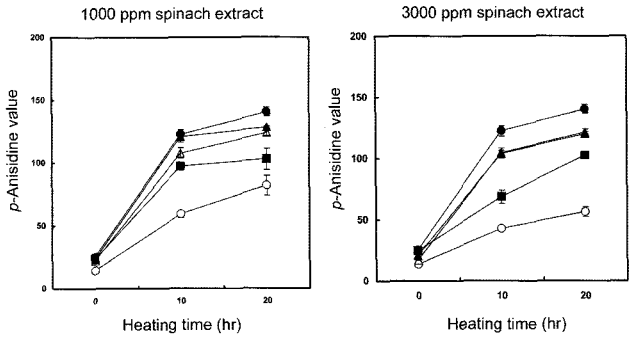

Fig. 3. *p*-Anisidine values of spinach extract-added lard during heating at 180°C for 20 hr. ●, No additives in lard; ○, *n*-Hexane extract; ▲, Ethyl ether extract; △, Ethyl acetate extract; ■, Ethanol extract.

of FFA could give an adverse effect on the lard oxidation; FFA accelerates the lipid oxidation by decreasing the surface tension of the oil and increasing the diffusion rate of oxygen from headspace to the oil (17).

Figure 3 shows PAV changes in lard during heating at 180°C for 20 hr. PAV of lard rapidly increased as the heating time increased from 0 to 10 hr, and a slight change in PAV was observed after 10 hr heating. This tendency was similar to that of soybean oil during frying of carrot-added flour dough (18). PAV of lard added with spinach extracts was lower than that of the lard without spinach extracts, indicating that spinach extracts decreased the formation of secondary oxidation products in lard during heating at 180°C. Zandi and Gordon (6) reported that methanol extract of old tea leaves lowered PAV of rapeseed oil during heating at 180. Lard added with hexane extract of spinach showed the lowest PAV throughout the heating time, and lower PAVs were

Table 3. Fatty acid compositions of lard added with 1,000 ppm SACC fractions of hexane-extract of spinach extract during heating at 180°C for 20 hr

Additive (hexane: benzene)[1)]	Heating time (hr)	Relative content (%)					P/L[2)]
		C16:0	C16:1	C18:0	C18:1	C18:2	
None	0[3)]	25.40±0.72[4)]	2.57±0.03	11.73±0.84	43.87±1.19	12.17±0.21	2.09
	10	26.91±0.50	2.59±0.02	13.44±0.06	43.18±0.03	9.99±0.18	2.69
	20	28.98±1.27	2.59±0.17	14.27±2.12	42.56±2.49	7.95±0.64	3.65
Fr 1 (100:0)	0	20.57±2.19	2.54±0.17	9.06±2.23	50.49±3.49	13.97±1.00	1.47
	10	26.16±0.84	2.62±0.31	11.96±2.10	44.82±1.12	10.63±0.63	2.46
	20	27.33±1.58	2.64±0.15	13.24±2.07	44.45±2.71	8.49±0.55	3.22
Fr 2 (95:5)	0	20.65±0.72	2.82±0.16	8.19±0.79	50.18±0.54	14.05±0.24	1.47
	10	25.66±1.16	2.76±0.00	10.88±0.87	46.22±1.58	10.62±0.58	2.42
	20	26.88±0.71	2.52±0.20	13.51±0.74	46.14±0.79	7.95±0.14	3.38
Fr 3 (90:10)	0	24.53±2.09	2.63±0.17	10.98±1.98	45.23±2.89	12.69±0.88	1.93
	10	25.62±0.09	2.68±0.10	11.50±0.48	45.19±0.91	10.81±0.63	2.37
	20	27.41±0.63	2.68±0.07	12.85±0.32	44.52±0.34	8.44±0.48	3.25
Fr 4 (80:20)	0	23.59±0.78	2.82±0.09	8.77±0.65	47.38±1.08	13.25±0.30	1.78
	10	23.67±1.19	2.53±0.19	11.20±0.79	46.48±0.90	12.11±0.17	1.95
	20	26.36±1.29	2.63±0.10	12.50±0.21	44.84±0.31	9.45±1.35	2.79
Fr 5 (50:50)	0	22.01±3.33	2.84±0.26	9.00±2.82	48.24±4.33	13.46±1.41	1.64
	10	24.88±2.79	2.60±0.06	12.10±1.90	45.28±3.90	11.31±1.08	2.20
	20	28.01±1.44	2.46±0.27	14.76±2.63	42.22±3.41	8.67±0.39	3.23
Fr 6 (0:100)	0	25.04±0.65	2.61±0.09	11.33±0.39	44.36±0.89	12.44±0.13	2.01
	10	25.26±0.37	2.74±0.12	11.31±0.48	45.68±0.67	11.01±0.13	2.29
	20	26.71±0.33	2.62±0.08	12.89±0.83	44.37±0.99	9.55±0.19	2.80

[1)]Eluting solvent composition (v/v) in silicic acid column chromatography.
[2)]P/L: Content ratio of palmitic acid to linoleic acid.
[3)]Values at zero heating time means ones of samples analyzed immediately after temperature of the lard reached at 180°C.
[4)]Mean ± standard deviation.

observed in 3000 ppm hexane extract-added lard than in 1000 ppm hexane extract-added lard. These results clearly indicate that hexane extract of spinach decreased the lard oxidation during heating at 180°C for 20 hr.

Effects of SACC fractions of hexane extract of spinach on the oxidation of lard during heating The hexane extract of spinach was further separated into six fractions by SACC, and the yields were 7.8, 10.2, 3.7, 2.5, 6.7, and 3.5% when the volume ratios of benzene in the eluting solvent were 0, 5, 10, 20, 50, and 100%, respectively. Fatty acid compositions of lard added with SACC fractions of hexane extract of spinach at 1000 ppm during heating at 180°C are shown in Table 3. During heating, relative content of linoleic acid in lard decreased and that of palmitic acid increased, and the P/L ratio increased. The rate of P/L ratio increase with heating time was significantly low in lard added with SACC fractions 3, 4, and 6 of hexane extract of spinach.

CDA contents of lard added with 1,000 ppm SACC fractions of hexane extract of spinach during heating at 180°C for 20 hr are shown in Fig. 4. As heating time increased from 0 to 20 hr, CDA contents of lard without spinach extracts increased from 1.17 to 3.13%. This is partly due to isomerization of linoleic acid in lard into a more stable conjugated diene system. Addition of SACC fractions of hexane extract of spinach generally lowered the CDA values of lard during heating. Among SACC fractions, fraction 4 showed the highest effect on lowering CDA values of lard during heating.

Figure 5 shows PAV changes in lard added with 1,000 ppm SACC fractions of hexane extract of spinach during heating at 180°C for 20 hr. PAV of lard without any SACC fractions of spinach extracts rapidly increased from 19.9 to 133.2 as the heating time increased from 0 to 10 hr, and a slight change in PAV was observed after 10 hr heating. PAV of lard added with SACC fractions 3 and 4 of hexane extract of spinach was significantly lower than that of the lard without SACC fractions, indicating that SACC fractions 3 and 4 of hexane extract of spinach decreased the decomposition of oxidized lard during heating at 180 °C. These results clearly indicate that SACC fractions 3 and 4 of hexane extract of spinach possess high anti-oxidant activity on lard during heating.

Separation of lard-antioxidative compound present in SACC fractions of hexane extract of spinach SACC fractions 3 and 4 of hexane extract of spinach gave one spot in TLC (R_f value = 0.60), suggesting that the fractions contained a single compound. The compound was analyzed by NMR spectroscopy, LC-MS spectrometry, and FT-IR spectrophotometry. Five main peaks were shown in the NMR spectrum of the compound (Fig. 6). Signals at 0.9 and 1.4 ppm were thought to indicate the presence of methyl and methylene protons in aliphatic groups, respectively (19). A signal at 1.8 ppm represents a proton in the methylene group adjacent to a double bond. Signals at 3.6 and 7.3 ppm were thought to be due to hydrogens bound to sugars and aromatic ring compounds (11, 20).

Figure 7 shows the LC-MS spectrum of the compound. Peaks at 692 m/z (M^+) and 510 (M^+-180) m/z in LC-MS spectrum confirmed the presence of sugars in the structure. A base peak at 339 suggests the possible presence of another sugar (21). Serial reduction of 14 (339, 325, 311, and 297 m/z) indicates the presence of methylene groups

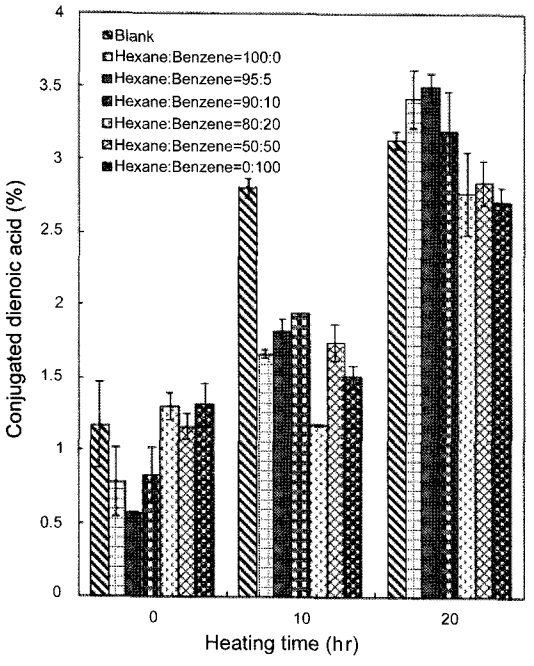

Fig. 4. Conjugated dienoic acid values of lard containing SACC fractions (1,000 ppm) of hexane extract of spinach during heating at 180°C for 20 hr. Values at zero heating time are for samples analyzed right after temperature of the lard reached at 180°C.

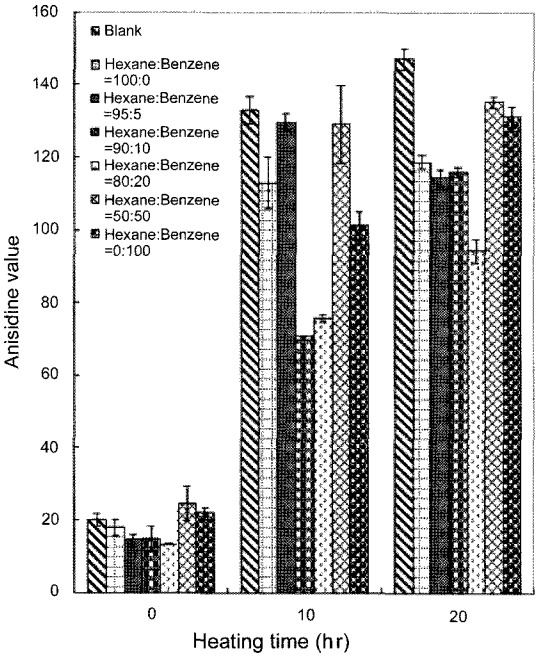

Fig. 5. *p*-Anisidine values of lard containing SACC fractions (1,000 ppm) of hexane extract of spinach during heating at 180°C for 20 hr. Values at zero heating time are for samples analyzed right after temperature of the lard reached at 180°C.

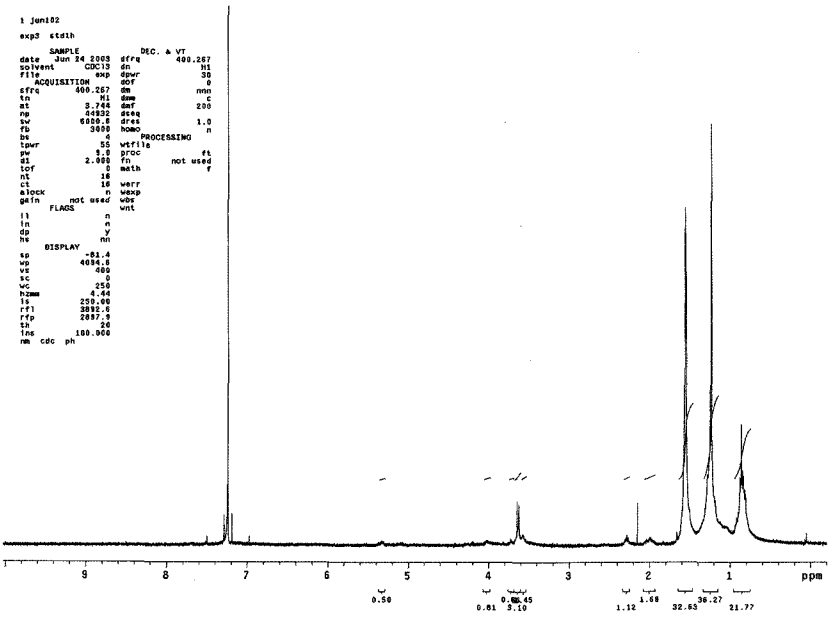

Fig. 6. ^{1}H-NMR spectrum of an antioxidant component from hexane extract of spinach.

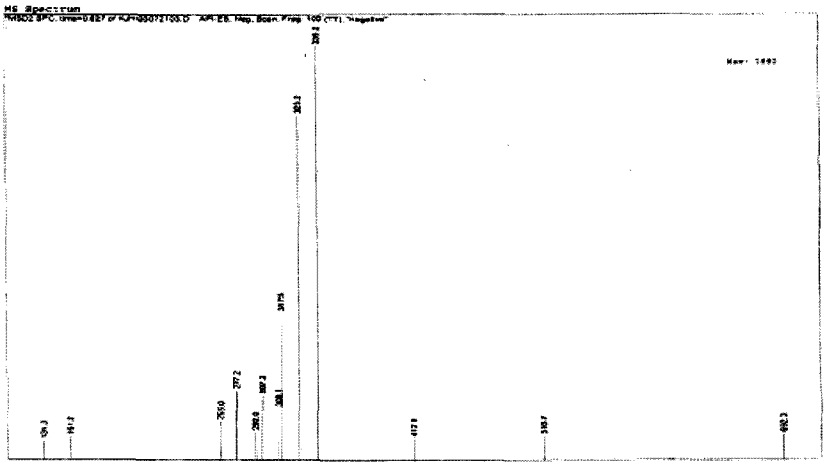

Fig. 7. LC-MS spectrum of an antioxidant component of hexane extract of spinach.

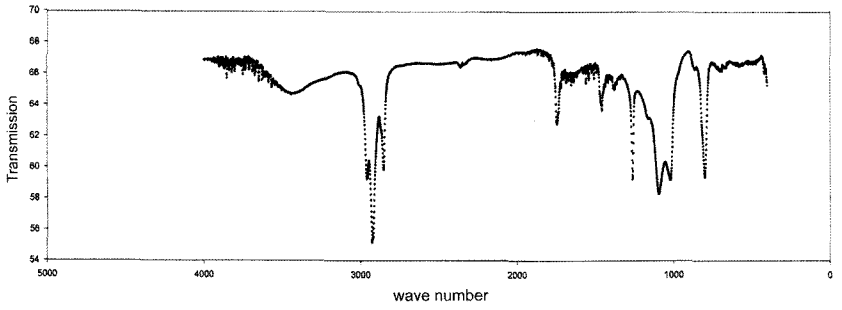

Fig. 8. FT-IR spectrum of an antioxidant component from hexane extract of spinach.

in the structure. Peaks at 265 and 309 m/z suggest the presence of carboxy group.

FT-IR spectrum of the compound (Fig. 8) shows the presence of a substituted benzene ring (700, 1000-1500 cm^{-1}), carbonyl group (1700-1800 cm^{-1}), alkane group (2850-3030 cm^{-1}), and hydroxy group (broad band between 3000 and 3500 cm^{-1}) in the structure (22). Although the exact structure of the compound having high antioxidant activity on lard during heating could not be determined, these results suggest that the antioxidant component separated by SACC and TLC from the hexane extract of spinach have sugar moieties and benzene ring along with

hydroxy, carbonyl, and alkyl groups in the structure.

Acknowledgments

This work was supported by a research grant from Inha University.

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