Journal of Chromatography B, 880 (2012) 42–49

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of bioactive compounds in fermented soybean products using GC/MS and further investigation of correlation of their bioactivities

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ARTICLE INFO

Article history:
Received 13 July 2011
Accepted 4 November 2011
Available online 17 November 2011

Keywords:
Soybean products
Fermentation
Active ingredients
Bioactivities
GC/MS

ABSTRACT

The active ingredients and bioactivities (anti-oxidant, anti-tyrosinase, anti-proliferative and estrogenic activities) of soybean and soybean products (Cheonggukjang, Meju, Makjang, Doenjang and soy sauce) produced by different fermentation processes were compared. There were high correlations between active ingredients and bioactivities. Free phenolic acids extracted from soybean products were identified and quantified by gas chromatography/mass spectrometry (GC/MS). Overall, the components and activities in fermented soybean products were different than those in soybeans. Total phenolic content (TPC), protein content (PC) and anti-oxidant activity increased as fermentation time increased. TPC and PC showed strong negative correlations with anti-oxidant activity. Doenjang and soy sauce, two long-term fermented products, showed lower total flavonoid content (TFC) and estrogenic activities than short-term fermented soybean products. This might be explained by the decomposition and hydrolysis of flavonoids due to the long fermentation time and high temperature. Strong anti-proliferative activity against cancer cell lines, which was highly correlated with TFC, was found in Meju and Cheonggukjang. Soybean and all fermented products except Meju exhibited effective tyrosinase inhibitory activities. Fermented products showed stronger estrogenic activity than soybeans, which was highly correlated with syringic acid.

1. Introduction

The soybean has been widely used for a long time as a healthy food and fermented soybean products are important components of traditional diets in Asian countries, such as Korea, China and Japan. In Korea, several traditional fermented soybean products such as Cheonggukjang, Makjang, Kochujang, Doenjang and soy sauce are the most commonly consumed goods (Fig. 1 for the fermentation conditions of these products).

Soybean contains various bioactive phytochemicals such as phenolic acids, flavonoids, isoflavones, saponins, phytosterols and sphingolipids [1–3] and possesses excellent immune-active effects in the human body [4]. The anti-oxidant activity can be caused by the phenolic compounds in soybean products [5,6]. Soybean isoflavones have multiple beneficial health effects, especially on estrogen-deficiency conditions such as menopausal symptoms [7] and exhibit high anti-tyrosinase activity [8]. Epidemiological data show that phytoestrogens in food, such as ones found in soy, can decrease the incidence rate of cancer in women [9]. Of course, the proportion of components and the bioactivities of soybean products might change during fermentation due to the different fermentation conditions. Cheonggukjang is considered to be healthier than soybeans [10]. Phenolic acid levels were shown to be increased after fermentation [11]. During the fermentation of Cheonggukjang, its isoflavone, flavanols and phenolic contents change [12]. Fermented soybean products exhibited stronger anti-oxidant activity than non-fermented ones [13,14]. Doenjang possesses much higher anti-cancer activity and anti-metastatic activity than soybeans [15]. Although many previous studies have reported the components and bioactivities of soybean products produced by different fermentation processes, there has been no systematic comparison of the active ingredients and bioactivities of the raw materials, intermediate products and final products obtained during soybean fermentation.

In this study, we report on variations between soybeans and fermented soybean products throughout the entire fermentation process. We investigated the contents of active ingredients

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doi:10.1016/j.jchromb.2011.11.013

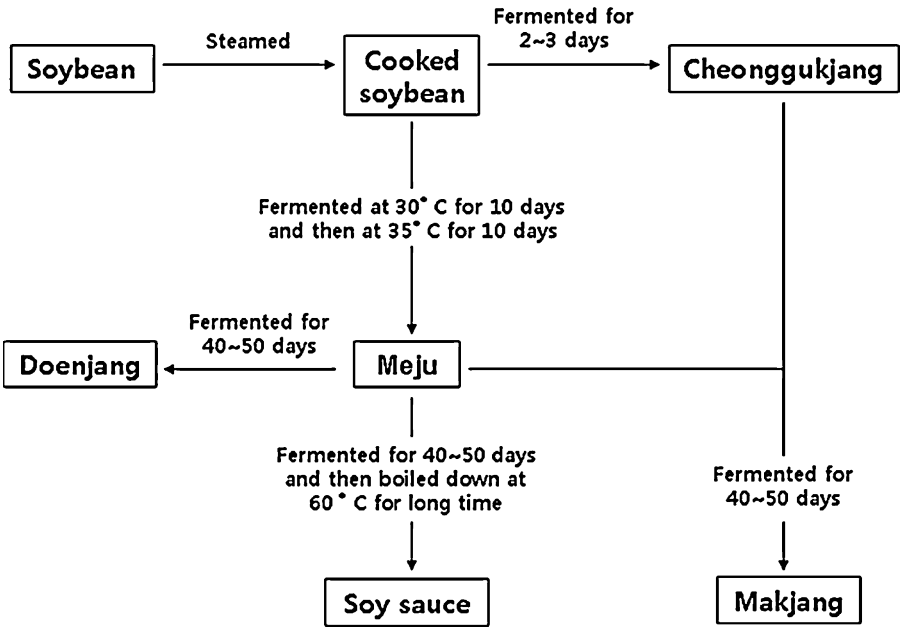

Fig. 1. Different fermentation conditions of soybean products (provided by Kongsalim, Korea).

including total phenolic acid content (TPC), total flavonoid content (TFC) and protein content (PC) in soybean products. Eight phenolic acid compounds (vanillic acid, protocatechuic acid, *m*-coumaric acid, syringic acid, *p*-coumaric acid, gallic acid, ferulic acid and caffeic acid) were also identified and quantified by GC/MS (gas chromatography/mass spectrometry). In addition, we compared bioactivities such as anti-oxidant activity (free radical scavenging activity), tyrosinase inhibitory activity, anti-proliferative activity and estrogenic activity *in vitro*. Furthermore, we investigated the high correlation between active ingredients and bioactivities of soybean products.

2. Materials and methods

2.1. Chemicals and reagents

Soybeans (SB) and soybean products [soy sauce (SS), Cheonggukjang (CJ), Meju (ME), Doenjang (DJ), Makjang (MJ), Fig. 1] were purchased from Kongsalim (Gyeongsangnam-do, Korea). Human breast cancer (MCF7), melanoma (B16) and human lung cancer (A549) cell lines were obtained from the Korean Cell Line Bank (KCLB, Cancer Research Institute, Seoul, Korea). RPMI Medium 1640, Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum, penicillin/streptomycin and all other required cell culture materials were purchased from Gibco-BRL (New York, NY, USA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

Soybeans and soybean products were dried, powdered using an electric mill (Shinil SFM-555SP, Hwasung, Korea) and sieved through 300-μm sieves. Approximate 10 g of the ground powder was dissolved in 100 mL of 80% methanol and then sonicated at room temperature for 15 min. The mixture was vortexed for 1 min and then centrifuged at 840 × *g* for 10 min. The above extraction process was repeated three times. Three extraction supernatants were combined, transferred into round flasks and then concentrated under reduced pressure. The extracts were weighed, dissolved in 20 mL of 80% methanol and then diluted for subsequent experiments.

To obtain the free phenolic acid fractions of soybean products, the samples were extracted according to the method of Krygier et al. [16] as shown in Fig. 2. Briefly, 10 g of ground powder was extracted three times with a 70% methanol/70% acetone (1:1, v/v) mixture. The combined supernatants were acidified to pH 2 and extracted with hexane. The aqueous phase was further extracted with a diethyl ether/ethyl acetate (DE/EA, 1:1, v/v) mixture. The organic layer was dehydrated and then concentrated under reduced pressure. The extract was weighed, dissolved in 5 mL of methanol and then dried by nitrogen purge.

2.3. Determination of total phenolic content (TPC)

TPC was measured by the Folin–Ciocalteu method [17] with slight modifications. Each test sample (30 μL) was added to 96-well microplates, followed by addition of 150 μL of Folin–Ciocalteu's phenol reagent. After approximately 8 min, 120 μL of 7.5% Na_2CO_3 was added to stop the reaction. The mixture was shaken gently and placed at room temperature for 60 min. The absorbance of the mixture was measured at 765 nm by an ELISA plate reader and compared to a standard curve based on prepared gallic acid solutions (25, 50, 100, 200 and 400 μg/mL). TPC was expressed as gallic acid equivalents (GAE).

2.4. Determination of total flavonoid content (TFC)

TFC was determined using the aluminum chloride method [18]. First, 30 μL of 5% $NaNO_2$ was added to the samples (100 μL) and 5 min later, 30 μL of 10% $AlCl_3$ was added. The solution was mixed; after approximately 6 min, 300 μL of 1 mol/L NaOH and 510 μL of deionized distilled water were added to the tube. Then the absorbance was measured at 510 nm by an ELISA plate reader and compared to a standard curve of prepared quercetin solutions (25, 50, 100, 200 and 400 μg/mL). The total flavonoid content was expressed as quercetin equivalents (QE).

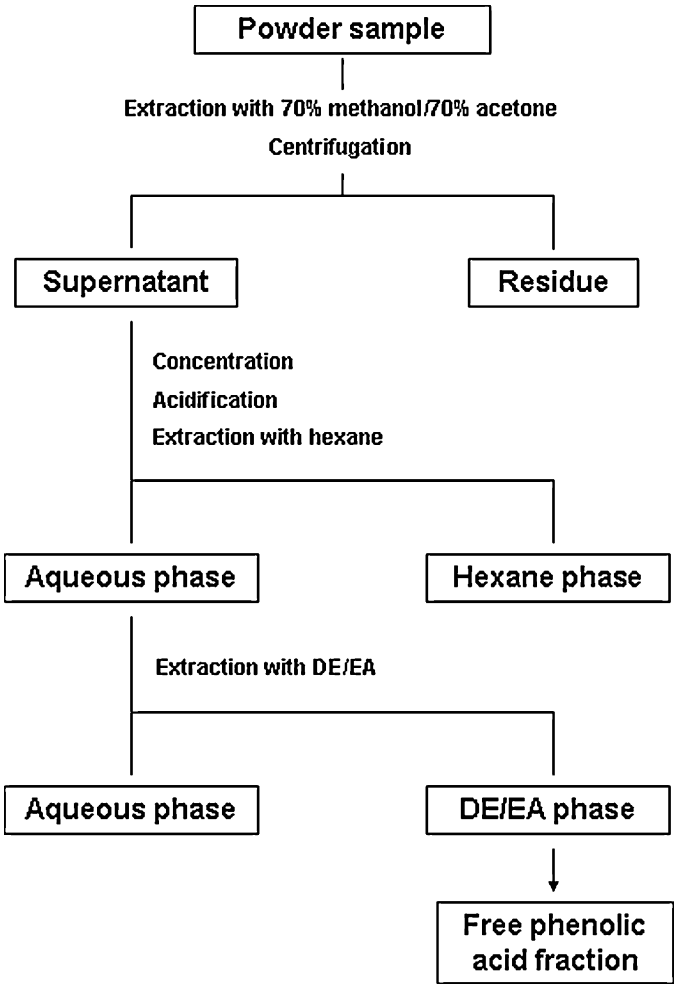

Fig. 2. Extraction procedure for the free phenolic acid fraction. DE/EA, diethyl ether/ethyl acetate.

2.5. Determination of protein content (PC)

PC in soybean products was estimated using a slightly modified Lowry protein assay [19]. Briefly, 1 mL of cupri-tartaric solution and 0.2 mL of test sample were mixed in a test tube and then incubated with 0.1 mL of 50% Folin–Ciocalteu's phenol reagent for 30 min at room temperature. Then the absorbance was measured at 750 nm by an ELISA plate reader. The measurement was compared to a standard curve of bovine serum albumin in deionized distilled water (0.0625–1 mg/mL). The protein content was expressed as bovine serum albumin equivalent (BE).

2.6. DPPH radical scavenging activity

The anti-oxidation assay was adapted from a previously described method [20]. The radical scavenging activity of soybean products was calculated according to the equation:

$$\text{DPPH radical scavenging activity}\ (\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100,$$

where A_{control} was the absorbance of the blank solution and A_{sample} was the absorbance in the presence of the test sample. The inhibition concentration (IC_{50}) was calculated as the concentration required for 50% inhibition of scavenging ability. Results were compared with the scavenging activity of ascorbic acid as a positive control.

2.7. Tyrosinase inhibitory activity

The tyrosinase inhibitory assay was performed as described by Piao et al. [21]. Inhibition of tyrosinase activity was calculated by the following equation:

$$\text{Tyrosinase inhibition activity}\ (\%) = \left[1 - \left(\frac{A_{\text{sample\_a}} - A_{\text{sample\_b}}}{A_{\text{control\_a}} - A_{\text{control\_b}}}\right)\right] \times 100,$$

where $A_{\text{control\_a}}$ was the absorbance of blank solution after incubation, $A_{\text{control\_b}}$ was the absorbance of blank solution before incubation, $A_{\text{sample\_a}}$ was the absorbance of sample solution after incubation and $A_{\text{sample\_b}}$ was the absorbance of sample solution before incubation. The results were compared to that of arbutin as a positive control.

2.8. Cell culture

MCF7, B16 and A549 cell lines were maintained in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. The Ishikawa cell lines were grown in DMEM/F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Cells were plated on a 175 cm^2 surface area in a 5% CO_2 incubator at 37 °C. The culture medium was changed every 2–3 days for subsequent experiments.

2.9. Anti-proliferative activity

Anti-proliferative ability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) colorimetric assay [22]. In brief, cancer cell lines, including MCF7, B16 and A549 cell lines were harvested with 0.25% trypsin and plated in 96-well microplates with RPMI1640 medium at a density of 8×10^3 cells/well overnight. After 24-h incubation to allow for cell attachment, the cell lines were treated with various concentrations of test samples in medium (200 μL) and incubated for 48 h in a 5% CO_2 incubator at 37 °C. The treated cells were washed with PBS and 200 μL of MTT solution (5 mg/mL MTT stock in PBS diluted to 0.5 mg/mL with RPMI1640 medium) was added to each well. The plates were incubated for 4 h in a 5% CO_2 incubator at 37 °C. Insoluble formazan crystals in the viable cells were dissolved in 100 μL DMSO after removing the medium containing MTT and the absorbance was measured at 570 nm by an ELISA plate reader.

2.10. Estrogenic and anti-estrogenic activities

The Ishikawa cell lines were used to test the estrogenic activities and anti-estrogenic activities of the samples [23]. For the estrogenic activity test, the medium was replaced with fresh estrogen-free medium containing different concentrations of test samples. To assess the anti-estrogenic activities, the medium was changed to fresh estrogen-free medium containing 1 nM estradiol (E_2) and test samples in a final volume of 200 μL. After a 72-h incubation, the cells were washed with PBS and 50 μL of 0.1% Triton X-100 (v/v) in 0.1 M Trizma buffer (pH 9.8) was added to each well. After incubation for 60 min, 150 μL of *p*-nitrophenyl phosphate solution was added to the cells and the activity was monitored at 405 nm every 14 s (22 readings) on an ELISA plate reader. Cell cytotoxicity was also determined by the MTT colorimetric assay.

2.11. GC/MS analysis of soybean products

Free phenolic acid fractions were analyzed on an Agilent 6890 Series Plus gas chromatograph (Agilent Technologies, USA)

Table 1
Total phenolic content (expressed as mg GAE/100 g dry weight), total flavonoid content (expressed as mg QE/100 g dry weight) and protein content (expressed as mg BE/g dry weight) in soybean products. Data represent mean ± SD ($n = 3$). SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce.

	SB	CJ	ME	MJ	DJ	SS
TPC (GAE mg/100 g)[a]	197 ± 3.3	735 ± 1.9	415 ± 3.5	1261 ± 4.3	942 ± 1.9	1062 ± 5.5
TFC (QE mg/100 g)[b]	178 ± 0.7	259 ± 4.0	225 ± 4.2	231 ± 6.5	209 ± 1.4	208 ± 6.1
PC (BE mg/g)[c]	549 ± 36.2	5691 ± 9.1	1580 ± 28.5	9799 ± 55.4	7724 ± 13.8	10,211 ± 42.1

[a] Gallic acid equivalent mg per 100 g of dry weight.
[b] Quercetin equivalent mg per 100 g of dry weight.
[c] Albumin from bovine serum equivalent mg per g of dry weight.

equipped with a JMS-GC mate (JEOL, Japan) mass spectrometer. Samples were pretreated via trimethylsilylation at 37 °C for 30 min with 100 μL of BSTFA [bis(trimethylsilyl)trifluoroacetamide] reagent. The derivatized samples were separated by a DB-5 capillary column (60 m × 0.25 mm I.D., 0.25-μm film thickness, Hewlett Packard, USA). The column temperature was held at 100 °C for 2 min and then increased to 270 °C at a rate of 5 °C/min. The temperature was maintained at 270 °C for 6 min and then increased to 320 °C at a rate of 30 °C/min, the temperature was maintained at 320 °C for 10 min. The temperature of the ion source and the transfer line was 280 °C; the inlet temperature was 280 °C. Helium was used as the carrier gas and the flow rate was 1.0 mL/min.

2.12. Statistical analysis

All data represent mean ± SD (standard deviation). The values of bioactivities (IC_{50}, LC_{50} and EC_{50}) were obtained from logistic regression analysis. Correlations between active ingredients and various bioactivities of soybean products were statistically analyzed as follows:

Let X_{ij} and Y_{ij} be the jth repeatedly measured active ingredient and bioactivity, respectively. We modeled them as $X_{ij} = X_i + U_{ij}$ and $Y_{ij} = Y_i + V_{ij}$, where X_i and Y_j were the average active ingredient and bioactivity of the ith soybean product, U_{ij} and V_{ij} were measurement errors. The measurement errors, U_{ij} and V_{ij} were IID from $(0, \sigma_U^2)$ and $(0, \sigma_V^2)$, respectively. Here, $(0, \sigma^2)$ implied a distribution with mean 0 and variance σ^2. We were interested in the correlation coefficient ρ between X_i and Y_i. In this paper, we report two estimators of ρ, denoted by R and R^*. Here, R was the sample correlation of $(\bar{X}_1, \bar{Y}_1)$, where $\bar{X}_1$ and $\bar{Y}_1$ were sample mean of observations of the ith product. The simple estimator R was often slightly biased due to the measurement errors. The estimator R^* corrected the bias in R to define two estimators in detail, we let

$$S_{XY} = \frac{1}{n-1}\sum_{i=1}^{n}[(\bar{X}_1 - \bar{\bar{X}})(\bar{Y}_1 - \bar{\bar{Y}})],$$

$$S_{XX} = \frac{1}{n-1}\sum_{i=1}^{n}[(\bar{X}_1 - \bar{\bar{X}})(\bar{X}_1 - \bar{\bar{X}})],$$

$$S_{YY} = \frac{1}{n-1}\sum_{i=1}^{n}[(\bar{Y}_1 - \bar{\bar{Y}})(\bar{Y}_1 - \bar{\bar{Y}})],$$

$$S^*_{XX} = \frac{1}{n-1}\sum_{i=1}^{n}[(\bar{X}_1 - \bar{\bar{X}})(\bar{X}_1 - \bar{\bar{X}})] - \frac{1}{n}\frac{1}{m-1}\sum_{i=1}^{n}\sum_{j=1}^{m}(X_{ij} - \bar{X}_1)^2,$$

$$S^*_{YY} = \frac{1}{n-1}\sum_{i=1}^{n}[(\bar{Y}_1 - \bar{\bar{Y}})(\bar{Y}_1 - \bar{\bar{Y}})] - \frac{1}{n}\frac{1}{m-1}\sum_{i=1}^{n}\sum_{j=1}^{m}(Y_{ij} - \bar{Y}_1)^2.$$

Then, two estimators were defined by

$$R = \frac{S_{XY}}{\sqrt{S_{XX}S_{YY}}} \quad \text{and} \quad R^* = \frac{S_{XY}}{\sqrt{S^*_{XX}S^*_{YY}}}.$$

In below, we proceeded our discussion with R, which was more commonly used in practice due to its simplicity. However, we also reported R^* to help the understanding of readers.

3. Results and discussion

3.1. Active ingredients in soybean products

As soybean contains various bioactive phytochemicals, we compared the active ingredient contents in soybean products including total phenolic content (TPC), total flavonoid content (TFC) and protein content (PC). The results are summarized in Table 1. MJ had the highest TPC, followed by SS, DJ, CJ and ME. The TPC of SB was significantly lower than for the others. When TFC was measured, all 6 products showed similar levels. High PC levels were found in SS and MJ, followed by DJ, CJ, ME and SB.

As shown in Fig. 1, displaying how to make soybean products using the traditional method, soybeans are steamed first to prepare Cheonggukjang, a short-term (2–3 days) fermented soybean product. Cooked soybeans formed into blocks are fermented for 5–10 days to make Meju. Then the Meju is fermented for more than 40–50 days to make long-term soybean products such as soy sauce, Doenjang and Makjang with some differences (e.g., heat treatment or additives). Based on our results, the active contents of the soybean products changed during the fermentation process. TPC and PC of soybean products were increased as the fermentation time increased, whereas TFC showed relatively small variability among the products. Long-term fermented products such as DJ and SS showed lower TFC than the short-term fermented CJ and ME, which might result from extremely long fermentation periods and high temperatures, causing the flavonoids to decompose and hydrolyze. Our results are similar to those in previous studies [24].

3.2. Anti-oxidant activities of soybean products

The anti-oxidant activities of soybean products were measured as their DPPH free radical scavenging activities. As indicated in Table 2, the anti-oxidant activities of soybean products generally increased as the fermentation time increased. In correlation analyses of active ingredients and bioactivities (Table 3), the anti-oxidant capacities as IC_{50} values were negatively correlated with the TPC and PC. When the individual components in the free phenolic acid fractions were analyzed, the anti-oxidant activities were negatively correlated with vanillic acid and protocatechuic acid, and positively correlated with syringic acid, p-coumaric acid and ferulic acid. Based on these results, the anti-oxidant activities may be reasonably explained by the phenolic acid contents in soybean and

Table 2
Free-radical (1,1-diphenyl-2-picrylhydrzyl)-scavenging activities and tyrosinase-inhibition activities of soybean products. Data represent mean ± SD ($n = 3$). SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce.

IC_{50}[a] (mg/mL)	SB	CJ	ME	MJ	DJ	SS	Control
DPPH scavenging activity	33.7 ± 2.44	9.67 ± 0.24	15.4 ± 0.47	4.79 ± 0.03	5.69 ± 0.20	3.42 ± 0.12	0.03[b]
Anti-tyrosinase activity	0.001 ± 0.001	8.31 ± 0.09	657.9 ± 329.7	5.76 ± 0.45	8.12 ± 0.14	0.33 ± 0.02	0.14[c]

[a] Concentration showing 50% inhibition.
[b] Ascorbic acid was used as a positive control.
[c] Arbutin was used as a positive control.

Table 3
Correlations between various components and bioactivities of soybean products.

Correlations (r)	DPPH[i]	Anti-tyrosinase activity[j]	MTT (MCF7)[k]	MTT (B16)[l]	MTT (A549)[m]	Estrogenic activity[n]
TPC[a]	−0.898 (−0.899)	−0.425 (−0.444)	−0.623 (−0.721)	0.047 (0.054)	−0.392 (−0.394)	0.882 (0.888)
TFC[b]	−0.561 (−0.578)	0.195 (0.210)	−0.838 (−0.997)	−0.459 (−0.545)	−0.900 (−0.930)	0.721 (0.746)
PC[c]	−0.881 (−0.882)	−0.515 (−0.538)	−0.548 (−0.634)	0.085 (0.099)	−0.273 (−0.274)	0.839 (0.844)
Vanillic acid[d]	−0.656 (−0.657)	−0.496 (−0.518)	−0.189 (−0.218)	0.534 (0.618)	0.109 (0.110)	0.510 (0.513)
Protocatechuic acid[e]	−0.864 (−0.865)	−0.256 (−0.267)	−0.603 (−0.697)	−0.063 (−0.073)	−0.332 (−0.334)	0.713 (0.718)
Syringic acid[f]	0.976 (0.985)	0.321 (0.338)	0.765 (0.892)	−0.002 (−0.002)	0.525 (0.531)	−0.942 (−0.955)
p-Coumaric acid[g]	0.947 (0.948)	0.275 (0.287)	0.663 (0.766)	−0.219 (−0.254)	0.404 (0.406)	−0.842 (−0.847)
Ferulic acid[h]	0.917 (0.919)	0.011 (0.012)	0.738 (0.854)	−0.061 (−0.071)	0.524 (0.527)	−0.756 (−0.761)

+, positive correlation; −, negative correlation.
The value in parenthesis means R^*.
The correlations were calculated using IC_{50} (DPPH and anti-tyrosinase activity), LC_{50} (anti-proliferative activity) and EC_{50} (estrogenic activity).
[a] Total phenolic content in soybean products.
[b] Total flavonoid content in soybean products.
[c] Protein content in soybean products.
[d] Vanillic acid content in free phenolic acid fraction of soybean products.
[e] Protocatechuic acid content in free phenolic acid fraction of soybean products.
[f] Syringic acid content in free phenolic acid fraction of soybean products.
[g] *p*-Coumaric acid content in free phenolic acid fraction of soybean products.
[h] Ferulic acid content in free phenolic acid fraction of soybean products.
[i] Free radical scavenging activities of soybean products.
[j] Tyrosinase-inhibition activities at 20 mg/mL of soybean products.
[k] Cell anti-proliferation activities towards MCF7 cell line at 10 mg/mL of soybean products.
[l] Cell anti-proliferation activities towards B16 cell line at 10 mg/mL of soybean products.
[m] Cell anti-proliferation activities towards A549 cell line at 10 mg/mL of soybean products.
[n] Estrogenic activities at 200 μg/mL of soybean products.

soybean products, which agreed with the findings reported by a previous study [25].

3.3. Anti-tyrosinase activities of soybean products

The anti-tyrosinase activities of soybean products were examined using arbutin, an effective tyrosinase inhibitor, as a positive control (Table 2). All samples exhibited similar levels of high inhibitory activities comparable to the positive control, except for ME which exhibited no detectable activity from 5 mg/mL to 20 mg/mL. Previously isoflavones were reported to possess potent anti-tyrosinase activity [26]. We hypothesized that the observed anti-tyrosinase activity might have resulted from various components including isoflavones in soybean products. However, no correlation (shown in Table 3) was found between the activity and the compounds. Since it was shown that tyrosinase inhibitors can repress the conversion of tyrosine to dopa, dopaquinone and subsequently melanin, various tyrosinase inhibitors have been isolated and studied as potential candidates to decrease melanin content. Our results suggest that soybean products might be used as potential treatments of melanin-related disorders and as skin-whitening agents.

3.4. Anti-proliferative activities of soybean products

The anti-proliferative activities of soybean products against cancer cell lines including MCF7, B16 and A549 cell lines are illustrated in Fig. 3. The products displayed the strongest anti-proliferative activity against A549 cell line. All fermented products showed greater anti-proliferative activities, which increased in a dose-dependent manner, than the original soybeans and the short-term fermented ME and CJ, which contain high amounts of TFC, showed much stronger effects. The high negative correlation between TFC and the viability of the three kinds of cancer cells (Table 3) was consistent with previous studies [27], which reported that the high anti-proliferative activity could be explained by the high phenolic and flavonoid contents in samples. Therefore, we speculated that the anti-proliferative activities were mostly due to the high flavonoid contents in soybean products. Although fermentation increased the anti-cancer activity, as found for ME and CJ, the ultra-long fermentation time and high temperature can cause the flavonoids to decompose and hydrolyze, leading to declining anti-cancer activity. We propose that soybean products may serve as potential sources for food therapy due to their anti-proliferative ability.

3.5. Estrogenic and anti-estrogenic activities of soybean products

Estrogenic activities of soybean products were tested using estradiol (E_2) as a positive control. The estrogenic activities of soybean products in the Ishikawa human endometrial cell lines are shown in Fig. 4. From 50 μg/mL to 200 μg/mL, all the products except SB exhibited high estrogenic activities with no cytotoxicity (data not shown); CJ was slightly stronger than the others.

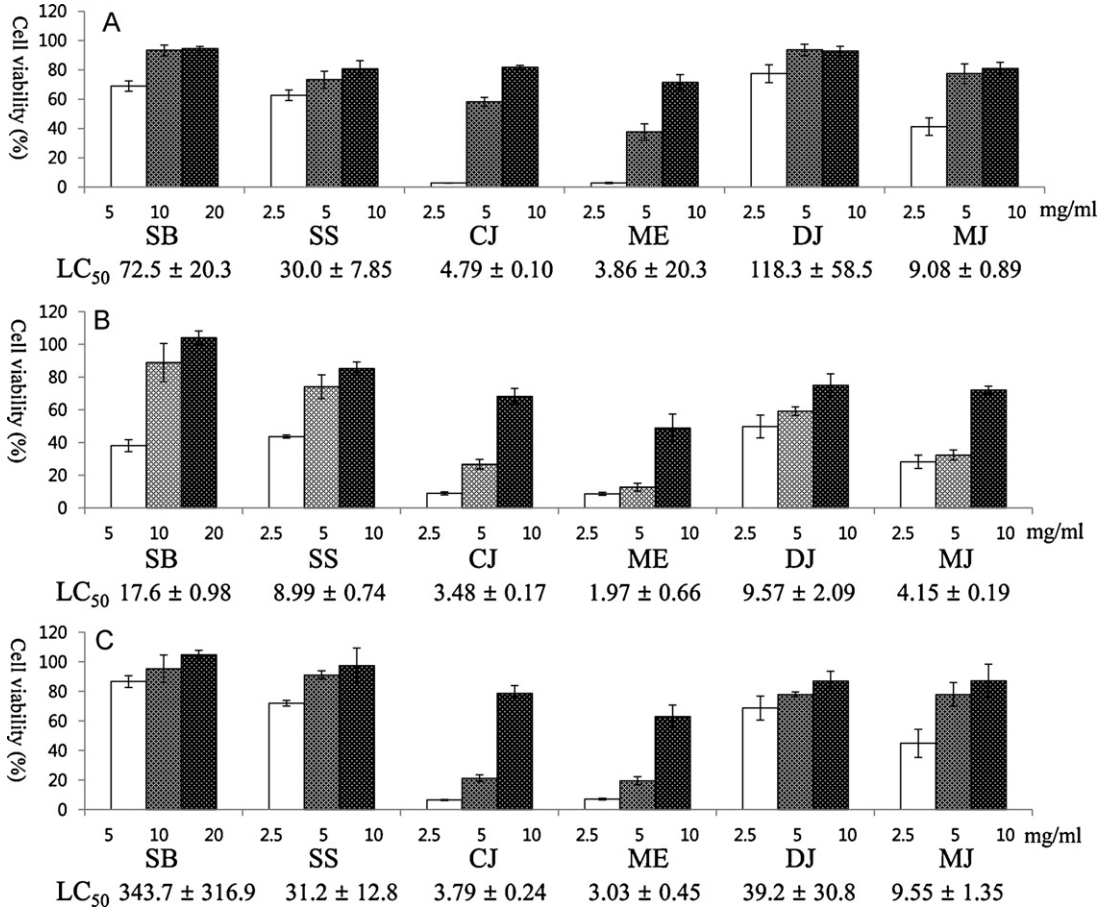

Fig. 3. Anti-proliferative activities of soybean products on B16 melanoma cell line (A), A549 human lung cancer cell line (B) and MCF7 breast cancer cell line (C). Data represent mean ± SD ($n = 3$). The 50% lethal concentrations of each cell line treated by soybean products are shown as LC_{50} values. SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce.

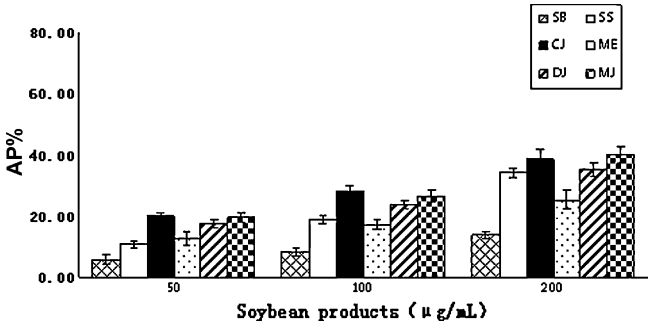

Fig. 4. Estrogenic activities of soybean products on Ishikawa human endometrial cell lines. Data represent mean ± SD ($n = 5$). SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce. AP%, relative alkaline phosphatase activity.

Besides, none of the soybean products showed any anti-estrogenic activity at the tested concentrations (data not shown). A previous study reported that soybean possessed the phytoestrogens known as isoflavones, which can exert estrogenic effects *in vivo* [28]. Therefore we supposed that the weaker estrogenic activities of SS and DJ might be explained by the hydrolysis of the isoflavones by long fermentation. The estrogenic activities of soybean products exhibited a strong positive correlation with TPC and PC and a slightly weaker correlation with TFC. In the analysis of individual free phenolic acids, they were highly negatively correlated with syringic acid, *p*-coumaric acid and ferulic acid, and positively correlated with protocatechuic acid (Table 3).

3.6. Analysis of free phenolic acids in soybean products by GC/MS

Considering the high correlations of TPC with anti-oxidant activity and estrogenic activity, we further investigated the free phenolic acids in the soybean products. Eight of the most abundant phenolic acid compounds (vanillic acid, protocatechuic acid, *m*-coumaric acid, syringic acid, *p*-coumaric acid, gallic acid, ferulic acid and caffeic acid) were chosen and their contents in the free phenolic acid fraction of soybean products were determined using GC/MS analysis. For the method validation, the linearity, accuracy, precision and the limit of quantification were examined and their values are summarized in Tables 4 and 5. Representative GC total ion chromatograms of the standard solution and various soybean product samples are shown in Fig. 5. Contents of the 8 free phenolic acids in soybean products are summarized in Table 6; vanillic acid, protocatechuic acid, syringic acid, *p*-coumaric acid and ferulic acid were identified in soybean products whereas the others were not found. Different anti-oxidant activities in various soybean products, which were strongly correlated with TPC, do not seem to be associated with the total amounts of the measured phenolic compounds. One possible explanation is that the changes in anti-oxidant activities in soybean products might not be caused by the 8 compounds

Table 4
Results of GC/MS method validation; related DPPH scavenging activities and anti-tyrosinase activities of 8 phenolic acids ($n = 3$).

Peak number	Standard	MW	Silylated compound	Retention time (min)	Regression equation[a]	r^2	LOD[b] (μg/mL)	LOQ[b] (μg/mL)	Precision (RSD[c] %)
1	Vanillic acid	168	312	23.57	$y = 4820x - 40{,}536$	0.9994	10	20	0.97
2	Protocatechuic acid	154	370	25.11	$y = 8350x - 29{,}570$	0.9987	2.5	10	1.34
3	*m*-Coumaric acid	164	308	26.05	$y = 5508x - 52{,}142$	0.9995	10	20	1.05
4	Syringic acid	198	342	26.45	$y = 4608x - 29{,}743$	0.9996	5	10	2.57
5	*p*-Coumaric acid	164	308	27.32	$y = 6957x - 53{,}581$	0.9989	5	10	1.90
6	Gallic acid	170	458	28.02	$y = 11{,}104x + 14{,}691$	0.9917	0.8	2.5	1.39
7	Ferulic acid	194	338	30.25	$y = 9318x - 100{,}461$	0.9993	10	20	2.31
8	Caffeic acid	180	396	31.16	$y = 15{,}395x - 45{,}879$	0.9947	2.5	5	1.44

[a] y, peak area; x, concentration (μg/mL).
[b] Values were expressed as $3.3\sigma/s$ and $10\sigma/s$ for LOD and LOQ, respectively, where s is the slope and σ is the standard deviation of the regression line.
[c] Relative standard deviation (%) = (standard deviation/mean) × 100 at concentration of 40 μg/mL ($n = 3$).

Table 5
GC/MS validation: precision, accuracy for phenolic acids with soybean products. SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce.

Peak number	Phenolic acid			Soybean products					
				SB	CJ	ME	MJ	DJ	SS
1	Vanillic acid	Accuracy[a] (%)		105.3 ± 1.85	110.0 ± 0.63	112.2 ± 1.20	114.5 ± 1.72	105.2 ± 1.55	110.4 ± 1.39
		Precision (RSD[b] %)	Intra	1.9	3.1	0.9	0.6	1.2	1.5
			Inter	4.8	7.3	4.5	9.3	9.6	9.2
2	Protocatechuic acid	Accuracy (%)		102.8 ± 1.30	106.7 ± 0.74	101.9 ± 1.08	107.6 ± 3.57	106.8 ± 0.71	107.5 ± 2.00
		Precision (RSD %)	Intra	NT[c]	2.1	1.0	1.7	2.0	3.3
			Inter	NT	2.9	4.7	8.4	8.7	7.7
3	*m*-Coumaric acid	Accuracy (%)		95.4 ± 2.21	103.0 ± 0.75	102.0 ± 0.74	107.1 ± 1.60	103.1 ± 0.68	103.5 ± 3.90
4	Syringic acid	Accuracy (%)		104.8 ± 3.57	104.1 ± 1.30	100.9 ± 1.51	110.5 ± 0.47	94.2 ± 1.15	108.1 ± 1.58
		Precision (RSD %)	Intra	3.0	2.5	1.3	3.3	3.6	2.3
			Inter	6.5	7.9	3.4	3.5	9.8	3.0
5	*p*-Coumaric acid	Accuracy (%)		94.8 ± 0.86	102.1 ± 1.88	98.4 ± 0.46	117.6 ± 2.74	117.1 ± 0.70	110.3 ± 0.60
		Precision (RSD %)	Intra	2.2	0.8	1.2	NT	NT	NT
			Inter	7.1	1.2	2.8	NT	NT	NT
6	Gallic acid	Accuracy (%)		92.7 ± 1.37	103.0 ± 1.07	102.4 ± 0.94	108.3 ± 1.48	108.6 ± 2.35	105.4 ± 0.39
7	Ferulic acid	Accuracy (%)		100.7 ± 2.57	108.7 ± 1.30	108.4 ± 1.59	104.2 ± 0.91	105.5 ± 1.64	102.5 ± 0.70
		Precision (RSD %)	Intra	1.1	2.9	0.7	1.7	2.1	2.0
			Inter	3.5	6.0	3.0	3.6	7.5	3.2
8	Caffeic acid	Accuracy (%)		94.7 ± 0.11	102.8 ± 1.22	98.8 ± 2.70	100.9 ± 0.38	101.3 ± 2.10	98.2 ± 1.25

[a] Calculated as [(found/nominal) × 100%] ± SD ($n = 3$) at a concentration of 100 μg/mL.
[b] Relative standard deviation (%) = (standard deviation/mean) × 100 ($n = 3$).
[c] Not tested.

Table 6
Compositions of free phenolic acids in soybean products (mg/kg of samples). Data represent mean ± SD ($n = 3$). SB, soybean; CJ, Cheonggukjang; ME, Meju; MJ, Makjang; DJ, Doenjang; SS, soy sauce.

Peak number	Phenolic acid	Soybean products (mg/kg)					
		SB	CJ	ME	MJ	DJ	SS
1	Vanillic acid	17 ± 0.3	15 ± 0.5	13 ± 0.1	41 ± 0.3	51 ± 0.6	46 ± 0.7
2	Protocatechuic acid	ND[a]	3 ± 0.1	3 ± 0.0	7 ± 0.1	5 ± 0.1	9 ± 0.3
3	*m*-Coumaric acid	ND	ND	ND	ND	ND	ND
4	Syringic acid	36 ± 1.1	30 ± 0.7	33 ± 0.4	29 ± 0.9	3 ± 1.1	28 ± 0.7
5	*p*-Coumaric acid	11 ± 0.2	5 ± 0.0	6 ± 0.1	ND	ND	ND
6	Gallic acid	ND	ND	ND	ND	ND	ND
7	Ferulic acid	23 ± 0.3	18 ± 0.5	15 ± 0.1	11 ± 0.2	1 ± 0.3	11 ± 0.2
8	Caffeic acid	ND	ND	ND	ND	ND	ND
	Total	88	71	70	88	98	94

[a] Not detected.

that were determined in this study, but by other components of TPC such as insoluble-bound phenolic acids and soluble ester forms. Therefore, research on more phenolic compounds is necessary to determine the correlations between individual compounds and their bioactivities. Moreover, in light of the high correlation of TFC with anti-proliferative and estrogenic activities, further identification of the flavonoids present in soybean products is needed.

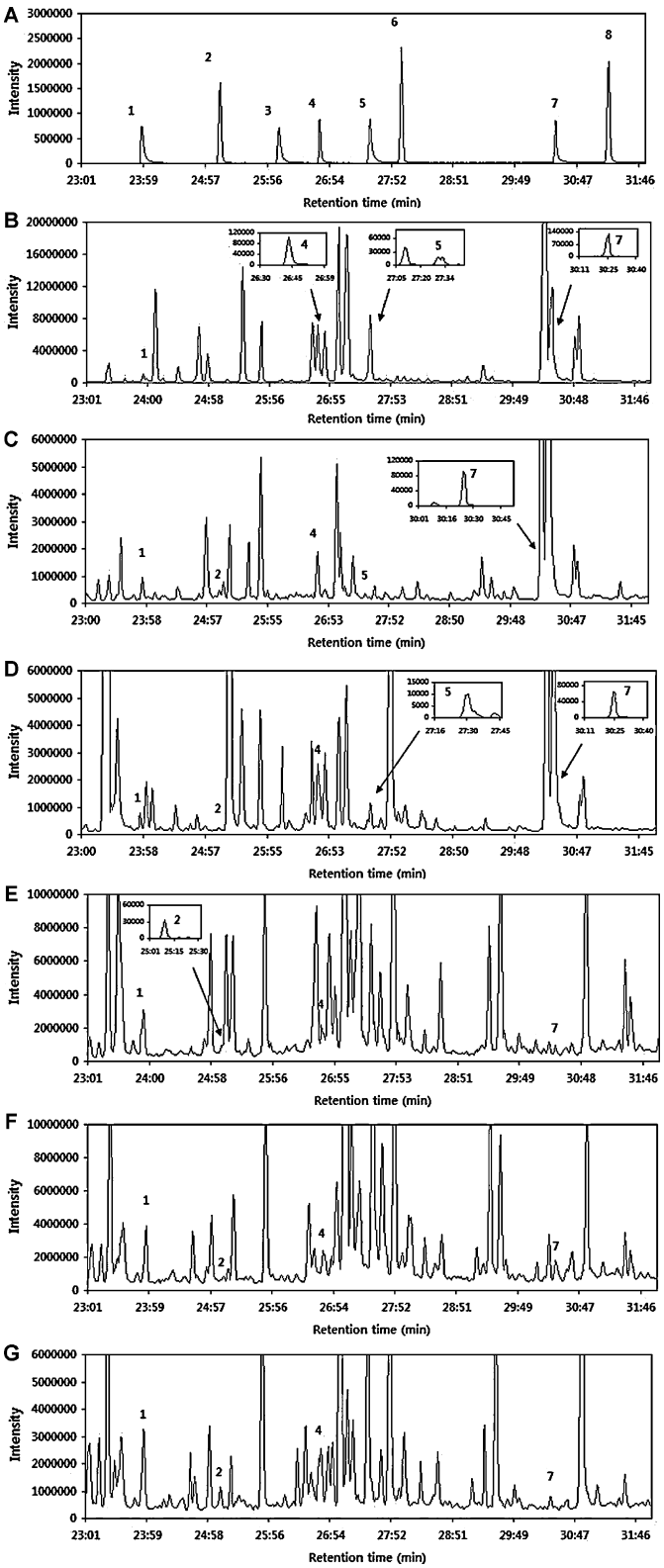

Fig. 5. Representative GC total ion chromatograms of 8 phenolic acid standards (A) and free phenolic acids in soybean products [soybean (B), Cheonggukjang (C), Meju (D), Makjang (E), Doenjang (F) and soy sauce (G)]; 1: vanillic acid; 2: protocatechuic acid; 3: *m*-coumaric acid; 4: syringic acid; 5: *p*-coumaric acid; 6: gallic acid; 7: ferulic acid; 8: caffeic acid. Extracted ion chromatograms of phenolic acid compounds are shown in inserted figures when the compounds are incompletely resolved.

4. Conclusions

In the present study, we measured the contents of active ingredients such as total phenolic content (TPC), total flavonoid content (TFC) and protein content (PC) in soybean (SB) and its fermented products, Cheonggukjang (CJ), Meju (ME), Makjang (MJ), Doenjang (DJ) and soy sauce (SS). We performed systematic comparison and correlation analysis of these ingredients and bioactivities including anti-oxidant activity, anti-tyrosinase activity, anti-proliferative activity and estrogenic activity. As the fermentation time increased, TPC, PC and anti-oxidant activity were increased, and TPC and PC showed high correlations with anti-oxidant activity. DJ and SS contained a lower TFC and lower estrogenic activities than short-term fermented soybean products, possibly because of the decomposition and hydrolysis of the flavonoids due to the long fermentation time and high temperature. ME and CJ exhibited strong anti-proliferative effects against A549 cells and they were highly correlated with TFC. All of the soybean products except ME exhibited effective tyrosinase inhibitory activities, which might be attributed to the presence of isoflavones among the active ingredients. A GC/MS method was developed and validated for the determination of 8 common free phenolic acids in soybean products. Although TPC and the individual phenolic compounds were strongly correlated with the anti-oxidant activity, total amounts of the measured phenolic compounds did not seem to be associated with the anti-oxidant activity of soybean products.

Acknowledgements

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008202), Rural Development Administration, Republic of Korea.

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