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Free-radical-scavenging and tyrosinase-inhibition activities of Cheonggukjang samples fermented for various times

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Abstract

Methanol extracts of Cheonggukjang, fermented for various times, were evaluated for their free-radical-scavenging and tyrosinase-inhibitory activities and the underlying mechanisms were elucidated. The free-radical-scavenging activity was highest for Cheonggukjang extracts fermented for 40 h and this decreased as the fermentation time shortened. The tyrosinase-inhibition activity increased with the length of fermentation. While levels of total phenolic compounds were highest in the 40-h-fermented Cheonggukjang samples, the total flavonoid content was lower compared to samples fermented for shorter times. Therefore, the antioxidative and tyrosinase-inhibition activities exhibited by methanol-extracted Cheonggukjang samples may be attributable to the contents of total phenolic compounds.

Keywords: Cheonggukjang; Free-radical-scavenging activity; Tyrosinase-inhibition activity; Total phenolic content; Total flavonoid content

1. Introduction

Cheonggukjang is a traditional Korean food that is produced by fermenting boiled soybeans with rice straw and it has a characteristic flavour, taste and nutritional composition (Kim et al., 2004). In addition to being consumed for its nutritional value, Cheonggukjang exhibits antioxidative, antimicrobial and many other beneficial bioactivities (Kim, Yang, & Song, 1999; Shon, Seo, Lee, Choi, & Sung, 2000; Youn, Choi, Hur, & Hong, 2001). The effects of Cheonggukjang on immune responses and gastrointestinal functions in rats have been reported by Lee, Yang, Song, Chai, and Kim (2006). An analysis of the chemical components of samples of Cheonggukjang from nine regions in Korea has revealed that Cheonggukjang contains many biochemical components, including fatty acids, amino acids, carbohydrates and organic acids (Kim et al., 1998). The isoflavone distribution and β-glucosidase activity in Cheonggukjang were investigated by Yang, Chang, and Lee (2006). The fermentation characteristics of *Bacillus* spp. with high protease activity extracted from Cheonggukjang have also been reported (Ahn, Kim, & Shin, 2006). A metabolomic analysis of Cheonggukjang during fermentation has been carried out using ^{1}H nuclear magnetic resonance spectrometry and principal component analysis (Choi, Yoon, Kim, & Kwon, 2007). However, the scavenging of free radicals and the inhibition of tyrosinase activity by Cheonggukjang samples at different fermentation times have not been investigated. Tyrosinase is a key enzyme for melanin biosynthesis in plants and animals, so inhibition of tyrosinase activity may be useful for the treatment of disorders associated with melanin hyperpigmentation (Masamoto et al., 2003). In this study,

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we examined the free-radical-scavenging and tyrosinase-inhibition activities of methanol extracts of Cheonggukjang fermented for different times. In addition, we measured the total phenolic and flavonoid contents in the extracts.

2. Materials and methods

2.1. Cheonggukjang samples

Samples of Cheonggukjang were obtained from Chunbuk National University. To summarise the traditional fermentation method, the white beans were submerged at 15 °C in water, for 15 h, before being steamed in a commercially available steamer (NK, Eunkwang Machinery, Korea) at a pressure of 1.7 kg/cm^2 for 30 min. Thereafter, the beans were allowed to cool to 50 °C. The beans were then fermented with rice straw at 42 °C for 40 h in a fermentation room. Samples of Cheonggukjang produced by the fermenting beans were obtained 0, 5, 10, 20 and 40 h after the start of the fermentation process. The samples of Cheonggukjang were freeze-dried and stored at −80 °C.

2.2. Reagents

All reagents including L-ascorbic acid, 1,1-diphenyl-2-picrylhydrzyl (DPPH) and reagent-grade methanol, were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

2.3. Extraction of Cheonggukjang samples

A 200 g portion of each of the Cheonggukjang samples, with different fermentation times, was extracted with 2 l of dichloromethane at room temperature for 12 h (repeated three times) and was then filtered through Whatman No. 4 filter paper (Whatman Internation Ltd., Maidstone, England). The residue was then extracted with 2 l of methanol as described above. The methanol was evaporated under reduced pressure.

2.4. Free-radical-scavenging activity

Antioxidant activity was measured based on the scavenging of the stable free radical, 1,1-diphenyl-2-picrylhydrzyl (DPPH), using a modified version of a previously described method (Shimada, Fujikawa, Yahara, & Nakamura, 1992). A sample of each fraction (25 μl), in 50% methanol, was added to 475 μl of a solution that contained DPPH. After 30 min, the absorbance was measured at a wavelength of 492 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (GENios Pro, TECAN, Austria). The free-radical-scavenging activity was expressed as follows:

$$\text{DPPH scavenging activity } (\%) = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the control sample and A_s is the absorbance of the test sample. L-ascorbic acid (0.0625, 0.125, and 0.250 g/l) was used as a positive control.

2.5. Tyrosinase-inhibition activity

Tyrosinase-inhibition assays were performed according to a modification of the method developed by Hearing (Hearing, 1987). Phosphate buffer (0.067 M) was prepared by adding 1.19 g of $Na_2HPO_4 \cdot 12H_2O$ to 50 ml of distilled water and the pH was adjusted to 6.8 using phosphoric acid. Samples of L-DOPA (25 μl and 50 μl) were added to a 50-μl aliquot of phosphate buffer. After mixing well, 25 μl of tyrosinase (100 units/ml) was added and the mixture was incubated for 10 min at 25 °C. The enzyme reaction was terminated by immersing the mixture in an ice bath. The absorbance of the resultant DOPA chromophore was measured at 492 nm.

2.6. Total phenolics content

The total phenolic content was determined using a modified Folin–Ciocalteu method (Singleton & Rossi, 1965). Each test sample (40 μl) was added to a test tube (1 ml volume) that contained 520 μl of distilled water. After vortexing the tubes, 40 μl of Folin–Ciocalteu's phenol reagent (Sigma) was added to each tube. The tubes were vortexed and 6 min later, 400 μl of 7% Na_2CO_3 was added to each tube. The tubes were vortexed again and then allowed to stand for 90 min at room temperature. Thereafter, the absorbance of each sample was measured against a blank at 750 nm using a spectrophotometer (Optizeu 2120UV, Mecasys, Korea). A calibration curve was constructed using 0.125, 0.250, 0.500 and 1.000 g/l gallic acid (Sigma) as a standard. The total phenolic content is expressed as milligrams of gallic acid per gram of dry extract.

2.7. Total flavonoid content

The total flavonoid content was determined using a modified version of the method described by Zhishen, Mengcheng, and Jianming (1999). Each test sample (100 μl) and 400 μl of distilled water were added to a volumetric flask (1 ml volume). Five minutes after adding 30 μl of 5% $NaNO_2$, 30 μl of 10% $AlCl_3$ was added. After 6 min, 200 μl of 1 mol/l NaOH was added and the volume was made up to 1 ml with distilled water. The absorbance of the solution was measured against a blank at 510 nm using a spectrophotometer (Optizeu 2120UV). A calibration curve was constructed using 0.125, 0.250, 0.500 and 1.000 g/l catechin (Sigma) as a standard. The total flavonoid content is expressed as milligrams of catechin per gram of dry extract. All measurements in this study were made in triplicate and the data are reported as the mean value ± one standard deviation.

3. Results and discussion

3.1. Free-radical-scavenging by Cheonggukjang fermented for different times

The scavenging of free radicals by the methanol extract of Cheonggukjang, for different fermentation times, was tested by measuring scavenging of the stable free radical, DPPH. The Cheonggukjang sample fermented for 40 h exhibited the highest free-radical-scavenging activity. The free-radical-scavenging ability of the 12.5 g/l Cheonggukjang methanol extract decreased monotonically as the fermentation time decreased, from 10.2% to 25.5% at 0 and 40 h of fermentation, respectively (Table 1). These results suggest that Cheonggukjang, at a later stage of fermentation, is a better source of antioxidants, substances that may inhibit cancer and the effects of aging.

3.2. Inhibition of tyrosinase activity by Cheonggukjang at different fermentation times

As indicated in Table 2, the rate of tyrosinase-inhibition of the 25 g/l sample increased monotonically with the fermentation time. The degree to which tyrosinase activity was inhibited by the 25 g/l methanol extract from the 40-h-fermented sample (82.6%) was similar to that produced by treatment with 0.25 g/l kojic acid (83.3%).

Our findings suggest that the methanol extract of Cheonggukjang, fermented for more than 20 h, might be effective for both inhibiting browning and promoting the whitening of skin.

3.3. Correlation of free-radical-scavenging activity and tyrosinase-inhibition activities with phenolics content

The total phenolic and flavonoid contents of samples of Cheonggukjang during fermentation are listed in Tables 3 and 4. The total phenolic and flavonoid contents of the samples increased and decreased monotonically, respectively, with the fermentation time. The phenolic content of 12.5 g/l Cheonggukjang ranged from 588 to 964 mg gallic acid/g dried fraction, and the flavonoid content ranged from 384 to 357 mg catechin/g dried fraction. Yang et al. (2006) reported that the consistent changes in isoflavone profiles of Cheonggukjang were not observed after fermentation for 36 h. The present study is the first demonstration that total flavonoid content in Cheonggukkang decreases as fermentation progresses.

The correlation coefficients for the correlation of free-radical-scavenging and tyrosinase-inhibition activities with the phenolic contents are listed in Table 5. The correlation coefficients were 0.91 and 0.98 for the total phenolic content versus free-radical-scavenging activity in Cheonggukjang samples at concentrations from 6.25 to 25 g/l, while the

Table 1
Free-radical (1,1-diphenyl-2-picrylhydrzyl)-scavenging activity (%) of methanol extracts of Cheonggukjang at various concentrations and fermentation times

Cheonggukjang concentration (g/l)	Fermentation time					Ascorbic acid[a]
	0 h	5 h	10 h	20 h	40 h	
6.25	11.1 ± 4.4	9.1 ± 2.9	12.5 ± 2.0	16.6 ± 1.4	20.4 ± 4.8	32.0 ± 0.4 (0.0625 g/l)
12.5	10.2 ± 0.4	8.6 ± 0.9	15.9 ± 0.6	23.2 ± 2.2	25.5 ± 1.3	62.2 ± 1.1 (0.125 g/l)
25	13.7 ± 3.7	15.8 ± 2.1	32.6 ± 1.3	41.6 ± 2.2	37.9 ± 1.6	79.0 ± 0.7 (0.25 g/l)

[a] Positive control.

Table 2
Tyrosinase-inhibition activity (%) of the methanol extracts of Cheonggukjang at various concentrations and fermentation times

Cheonggukjang concentration (g/l)	Fermentation time					Kojic acid[a]
	0 h	5 h	10 h	20 h	40 h	
6.25	69.9 ± 5.0	72.5 ± 2.0	78.0 ± 7.0	77.2 ± 0.0	77.3 ± 0.2	78.6 ± 0.5 (0.0625 g/l)
12.5	69.3 ± 3.9	76.6 ± 8.4	75.7 ± 0.3	80.2 ± 3.7	81.2 ± 2.2	81.3 ± 0.4 (0.125 g/l)
25	71.8 ± 6.4	75.2 ± 1.0	75.9 ± 5.5	81.3 ± 1.6	82.6 ± 0.2	83.3 ± 0.6 (0.25 g/l)

[a] Positive control.

Table 3
Total phenolic content (expressed as mg gallic acid/g dried fraction) of methanol extracts of Cheonggukjang at various concentrations and fermentation times

Cheonggukjang concentration (g/l)	Concentration (g/l)	0 h	5 h	10 h	20 h	40 h
6.25	6.25	229 ± 4.3	228 ± 3.5	271 ± 9.8	303 ± 7.6	318 ± 8.0
12.5	12.5	588 ± 10.4	612 ± 8.3	743 ± 7.9	874 ± 20.7	964 ± 31.1
25	25	1630 ± 13.6	1694 ± 8.3	2193 ± 10.8	2569 ± 41.8	2629 ± 56.2

Table 4
Flavonoid content of the methanol extracts of Cheonggukjang during 0, 5, 10, 20 and 40 h fermentation at 6.25, 12.5 and 25 g/l concentrations

Cheonggukjang concentration (g/l)	Concentration (g/l)	0 h	5 h	10 h	20 h	40 h
6.25	6.25	180 ± 1.6	176 ± 0.7	175 ± 0.4	175 ± 0.6	174 ± 0.3
12.5	12.5	384 ± 7.5	369 ± 1.8	361 ± 1.2	365 ± 1.7	357 ± 0.3
25	25	863 ± 38.9	794 ± 4.3	772 ± 11.2	773 ± 1.7	747 ± 4.6

Table 5
Correlation coefficients for (a) and (b)

(a) For correlation of free-radical-scavenging and tyrosinase-inhibition activities versus the total phenolic content of methanol extracts of Cheonggukjang at concentrations of 6.25, 12.5, 25 g/l

Cheonggukjang concentration (g/l)	Free-radical-scavenging activity	Tyrosinase-inhibition activity
6.25	0.91	0.70
12.5	0.98	0.73
25	0.96	0.89

(b) For correlations of free-radical-scavenging and tyrosinase-inhibition activities versus the total phenolic content of methanol extracts at different fermentation times with different concentrations of Cheonggukjang

Fermentation time (h)	Free-radical-scavenging activity	Tyrosinase-inhibition activity
0	0.78	0.80
5	0.90	0.18
10	0.99	0.41
20	0.99	0.74
40	0.99	0.75

correlation coefficients were 0.70 and 0.89 for total phenolic content versus tyrosinase-inhibition activity (Table 5a), which suggest that there is a strong correlation between free-radical-scavenging activity and the total phenolic content within a sample. In addition, the correlation coefficients were calculated based on the data of total phenolic contents versus free-radical-scavenging and tyrosinase-inhibition activities at the three different concentrations (6.25 g/l, 12.5 g/l, 25 g/l) which were treated as one set at different fermentation times and those ranged from 0.78 to 0.99 (Table 5b).

It has been reported that the antioxidant activity of plant materials strongly correlates with their content of the phenolic compounds (Velioglu, Mazza, Gao, & Oomah, 1998). In the present study, the flavonoid content was lowest in the methanol extract of 40-h-fermented Cheonggukjang samples, whilst the total phenolic content was particularly high (Table 3). Therefore, the higher free-radical-scavenging and tyrosinase-inhibition activities of the methanol extract of 40-h-fermented samples may be due to the higher amounts of phenolic compounds in those samples. The fermentation of Cheonggukjang was known to be conducted by various microorganisms such as *Bacillus subtilis*, *Bacillus licheniformis* and so on (Kim et al., 1999). Even though specific key enzymes for enhanced phenolic compound production during Cheonggukjang fermentation were not elucidated, it was assumed that phenolic compounds produced by various enzymes during fermentation played an important role in the increased antioxidant activity.

In conclusion, we found that the methanol extract of 40-h-fermented Cheonggukjang exhibited the highest degrees of free-radical-scavenging and tyrosinase-inhibition activities. In addition, the total phenolic content increased while the total flavonoid content decreased as fermentation progressed. These results suggest that Cheonggukjang extracts possess antioxidative and tyrosinase-inhibition activities (which have been indicated to prevent hyperpigmentation), that are attributable to phenolics other than flavonoids.

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