Food Sci. Biotechnol. Vol. 14, No. 6, pp. 828 ~ 831 (2005)

Food Science and Biotechnology

An Approach to Increase Vitamin D_2 Level in *Doenjang* (Fermented Soybean Paste) using Mushrooms

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Abstract The content of vitamin D_2, including its precursor ergosterol, was determined in some cultivated mushrooms to manufacture fortified *Doenjang* (Korean traditional soybean paste) with vitamin D by supplementation with mushroom. Ergosterol was the most abundant sterol in the mushrooms (50 to 140 mg/100 g dry weight) but the ergocalciferol portion made up only 0.065% (*Pleurotus eryngii*) to 2.5% (stipe part of *Lentinus edodes*, shiitake) of the total vitamin D_2 of each mushroom. Changes in these compounds in *L. edodes* caused by UV or solar irradiation were also evaluated. Ergocalciferol content in the pileus part of *L. edodes* went up to 424 μg/100 g dry weight and ergosterol levels reached 139.3 mg per 100 g dry weight at maximum levels. Ergocalciferol content increased about 50% when exposed to solar radiation and increased 377% with UV irradiation. These compounds level in *Doenjang* was enriched as much as supplied UV irradiated *L. edodes* powder to before fermentation, and the supplemented mushroom did not influence the palatability of *Doenjang*.

Key words: soybean fermentation food, *Doenjang*, mushrooms, ergosterol, ergocalciferol, vitamin D_2

Introduction

The D vitamins, which include D_2 (ergocalciferol) and D_3 (cholecalciferol), are known to stimulate intestinal calcium and phosphate transport, bone calcium mobilization, and other functions (1). They inhibit iron-dependent liposomal lipid peroxidation and also act as anticancer materials (2). Vitamin D is produced physiologically by UV irradiation through a precursor in the skin or must be obtained from the diet. As people age, they have decreased serum vitamin D levels, since the efficiency of provitamin D photoproduction decreases as a consequence of advancing age (1). Vitamin D deficiency can lead to human diseases such as rickets and osteomalacia, especially in the elderly and in children. Therefore, human beings have to take in vitamin D from food. However, vegetables, fruits, or nuts do not contain any detectable vitamin D, and meat and non-fatty fish contain only trace amounts (3, 4).

Ergosterol, also known as provitamin D_2, is a constituent cell membrane and key component of cell structure and function in fungi, yeasts, and plants. It is found in worthwhile amounts in basidiomycetes (mushrooms) and ascomycetes but scarcely found other microorganisms (5). Ergosterol can be converted artificially to ergocalciferol by UV irradiation or solar action. Vitamin D_2 is commercially more important than vitamin D_3 as a food additive for humans and livestock because of its ready availability from ergosterol, as well as the fact that it has effects equal to those of vitamin D_3 in the treatment of osteomalacia (6). *Doenjang*, is a traditional soybean-based fermented food in Korea that is used as a sauce. The consumption of this material has increased recently with the release of findings that *Doenjang* may have some beneficial effects to health like antioxidant, fibrinolysis, antimutant, and immune functions (7, 8). Therefore, the aim of this study was to screen content of ergocalciferol and ergosterol from various edible mushrooms for use as a vitamin D source, and to investigate the effect of irradiated mushrooms on vitamin D content in *Doenjang*.

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Received July 31, 2005; accepted September 26, 2005

Materials and Methods

Mushrooms *Lentinus edodes* fruit bodies were harvested on *Quercus variabillis*, *Agaricus bisporus* was grown on compost, *Pleurotus ostreatus* was obtained from rice straw or waste cotton, *Pleurotus eryngii* grew on sawdust of Douglas-fir (*Pseudotsuga menziesii*), and *Flammulina velutipes*, was harvested from sawdust of hardwood. Mycelia of *Armillaria mellea* were grown on saccharified malt extract medium. All samples were freeze-dried and then crushed prior to sieving with a 30 mesh sieve.

Irradiation Ten grams of freeze-dried mushroom powder were laid on each glass petri dish (i.d. 85 mm × 15 mm) for irradiation. The petri dishes were placed either 10cm from a source of artificial UV irradiation or outdoors for solar irradiation. Further UV irradiation was carried out by an UV lamp (254 nm, VL-6LC, France) for 24 hours at

room temperature in a darkroom.

***Doenjang* manufacturing** *Doenjang* was manufactured by traditional methods and obtained from Paekche Traditional Food Company located in Korea. As a starter culture, soybeans presoaked in water for 24 hours were boiled at 100 for 60 minutes. The beans were then dried in the shade for 2 months with rice-straw to inoculate them with microorganisms such as *Bacillus* sp., yeast, and acid-forming bacteria. To manufacture *Doenjang*, cooked soybeans and starter culture were mixed in the ratio of 6 to 4 with 10% NaCl, 50% moisture, and 1-3% irradiated mushrooms. This mixture was fermented in an earthenware pot for at least 3 months.

Vitamin D measurement Ergocalciferol and ergosterol were extracted and analyzed according to Mau *et al.* (9). Five grams of each sample were mixed with 4 mL of sodium ascorbate (17.5%, w/v distilled water), 50 mL of ethanol (95%), 10 μL of 50% KOH, and 1 mL of internal standard (100 μg cholecalciferol (Sigma Co.)/mL MeOH). The mixture was saponified at 78°C for 1 hour and was then partitioned with 15 mL of deionized water (aqueous phase), and 50 mL of diethyl ether (organic phase). The aqueous phase was then extracted 3 times with 10 mL of ethanol and 50 mL of *n*-pentane, with 50 mL of *n*-pentane, and finally with 20 mL of *n*-pentane. The organic phase pool was washed 3 times with 50 mL of KOH in ethanol, and washed with deionized water to neutrality. The organic layer was evaporated under vacuum conditions in the dark and redissolved in 3 mL of MeOH.

The vitamin D content was quantified with a HPLC (Shimadzu LC-10 Series). The mixture was separated on a Prodigy 5 ODS-2 column (4.6×250 mm, 5 μm) with a Supelco Discovery C_{18} (4.0×20 mm, 5 μm) at 30°C and a mobile phase of methanol/acetonitrile (25/75; v/v). The flow rate was 1.3 mL/minutes through a sample loop of 20 μL. UV detection was at 264 nm. All samples were filtered using a membrane filter (0.45 μm) before injection.

***Doenjang* analysis** The moisture content was determined by oven-drying at 105°C. To evaluate amino nitrogen and soluble carbohydrate, five grams of *Doenjang* were mixed with 100 mL of distilled water and homogenized using a homogenizer (Omni Co., USA) with a 20 mm (i.d.) generator probe. The mixture was centrifuged at 3,000 × g, 4 for 20 min and then the supernatant was filtered. The amount of amino nitrogen was evaluated by formol method (10). To evaluate soluble carbohydrate, 4 mL of 0.3 M TCA (trichloroacetic acid) was added into 6 mL of the supernatant, and allowed to stand for 30 min before centrifuging (8,500 × g, 20°C for 20 min). Finally, the content was quantified by phenol-sulfuric acid method (11). The color of *Doenjang* was measured by a CM3500d colorimeter (Minolta Co., Japan) and the alcohol content of the sample was determined by oxidation-reduction titration (10).

Statistical analysis The data was expressed as means ± SEM of triplicate. Nine trained panelists evaluated the bean paste, and preference scores for each *Doenjang* sample were rated on a scale from 1 (very poor) to 9 (very good) for acceptability. Statistical analysis was performed by means of ANOVA followed by Duncan's test ($p<0.05$).

Results and Discussion

Screening of vitamin D source from mushrooms Ergocalciferol and ergosterol levels of various mushrooms are shown in Table 1. The content of total vitamin D_2 including ergosterol was approximately between 17.6 and 139.7 mg/100 g dry weight. Ergosterol, provitamin D was abundantly distributed in dried fruit bodies or mycelia of the mushrooms, whereas ergocalciferol content was only 65-573 μg/100 g dry weight. *F. velutipes* contained the highest level of ergocalciferol, but its ergosterol level was significantly lower than in the other mushrooms. From an industrial point of view, the mycelium of *A. mellea* may be an important vitamin D_2 source. Not only does it contain considerable levels of ergocalciferol/ergosterol (305 μg/101 mg per 100 g dry weight), but it is more economical to cultivate than fruiting bodies: it's faster (12), takes up less space, and growth media is cheaper. However, despite these benefits, mycelia have not been used as vitamin source. It was probably because of problems like the difficulty of separation of fungus from growth media and lower mass production.

Ergocalciferol was also found to be abundantly distributed in both the stipe and pileus of *L. edodes* (362 and 424 μg/100 g dry weight, respectively), while ergosterol levels in the stipe were negligible. Mattila *et al.* (13-15) reported that vitamin D_2 levels in cultivated mushrooms *A. bisporus*, *P. ostreatus*, and *L. edodes* were below 1 μg/100 g dry weight and ergosterol levels were 654-679 mg/100 g dry weight. Mau *et al.* (9) also reported that ergocalciferol/ergosterol levels were 220 μg/27.4 mg per 100 g dry weight in *A. bisporus* and 216 μg/29.7 mg/100 g dry weight in *L. edodes*. The differences in vitamin D_2 levels between individual mushrooms were probably due to natural variations in climate, harvest season, and temperature (16).

Irradiation to increase level of ergocalciferol *L. edodes* powder was irradiated with UV or sunlight to

Table 1. Vitamin D_2 content of various cultivated mushroom sources (per 100 g dry weight)

Vitamin D	Fruit bodies								Mycelium
	Lentinus edodes (shiitake)			*Agaricus bisporus*		*Pleurotus ostreatus*	*Flammulina velutipes*	*Pleurotus eryngii*	*Armillaria mellea*
	Whole	Stipe	Pileus	Brown	White				
Ergocalciferol (μg)	406±16	362±14	424±10	264±13	290±13	289±8	573±37	65±10	305±30
Ergosterol (mg)	94.0±3.8	17.3±0.7	139.3±3.4	76.3±3.7	48.5±2.3	102.7±2.9	56.2±3.6	99.9±3.9	101.8±10.2
Total vitamin D_2 (mg)	94.4	17.6	139.7	76.6	48.7	103.0	56.8	100.0	102.1

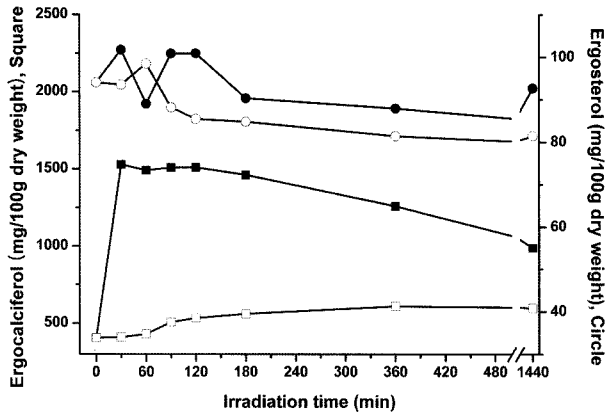

Fig. 1. Changes in ergocalciferol and ergosterol content in *Lentinus edodes* caused by UV or solar irradiation. Squares indicate ergocalciferol and circles represent ergosterol; black points=ultraviolet radiation, white points=solar radiation

increase the ergocalciferol value and the result is shown in Figure 1. After 30 minutes of UV irradiation treatment, ergocalciferol content increased about 377% and leveled off at 1529 μg/100 g dry weight for the next 90 minutes. Ergosterol content also increased slightly. However, the values for ergocalciferol decreased after 120 minutes and steadily declined up to 24 hours. On the other hand, Ergocalciferol value was increased moderately by solar irradiation, showing a gradual increase up to 50% that leveled off at 609 μg/100 g dry weight after 6 hours irradiation. Ergosterol content gradually decreased, however. As shown in Figure 1, artificial UV treatment was able to enrich ergocalciferol content, with optimal irradiation time between 30 minutes and 2 hours. After 2 hours, ergocalciferol levels were reduced by over-irradiation. Although it is well known that ergosterol can be converted to ergocalciferol by UV irradiation, the photosynthetic pathway for increasing ergosterol and the degradation pathway of ergocalciferol by over-irradiation are unclear. In the literature, ergocalciferol content of *L. edodes* could be enriched 2 to 6 fold for by UV irradiation 2 h (9,16), and ergosterol contents could also be increased (9). We assumed that UV irradiation caused the vitamin D_2 molecule to be transformed into a higher molecule by polymerization resulting from enzymatic and/or photo oxido-reductation of sterol.

Application to *Doenjang*

Chemical composition We investigated the chemical composition and aesthetic properties of *Doenjang* to know the effect of UV-irradiated *L. edodes* powder on product quality. As shown in Table 2, the color of *Doenjang* was an increasingly bright yellow as mushroom supplement concentration increased by 2%, since the mushroom powder was a much brighter color than it. However, the surface color at 3% was changed to dark than control. We assumed that the surface browning reaction may have been catalyzed during fermentation not only due to the increased surface area of *Doenjang* as a consequence of the addition of supplements but also due to the tyrosinase action of *L. edodes*. The browning reaction of *Doenjang* is mainly based on oxygen concentration, temperature and metal ion levels (17) all of which are related to amino-carbonyl and enzymatic reactions.

On the other hand, overall acceptability (Table 3) in palatability evaluation was high at 2%, although did not show significant difference ($p<0.05$). It is indicated that *L. edodes* did not affect quality. Amino nitrogen contents (Table 4) which affect taste and smell were slightly increased from 514 mg% to 587 mg% by supplementation of UV-irradiated *L. edodes*. In the literature, wild type of *L. edodes* contains 2.4% of these compounds (18) and the contents in *Doenjang* affected by microorganism during the fermentation (19). The effect of *L. edodes* on microorganism, related to these compounds is unclear, so further investigation is needed to understand this metabolic system.

Vitamin D_2 contents in *Doenjang* Table 5 shows that

Table 2. Changes in Hunter L, a and b values of *Doenjang* supplemented as affected by addition ratio of *L. edodes* powder

Addition ratio of mushroom powder to *Doenjang* (%)		L	a	b	ΔE[a]
Control (0%)	Surface	28.44	8.79	14.31	-
	Inside	32.80	12.43	20.03	-
1%	Surface	31.87	9.24	15.62	3.70
	Inside	32.22	12.44	19.18	1.03
2%	Surface	32.85	9.63	14.38	4.49
	Inside	33.86	12.76	20.95	1.44
3%	Surface	28.44	9.24	11.85	2.50
	Inside	34.65	12.82	20.58	1.97

[a]ΔE was calculated by equation $(\Delta a^2+\Delta b^2+\Delta c^2)^{1/2}$
L. edodes mushroom was irradiated under UV as described before fermentation for 30 min.

Table 3. Results on sensory evaluation of *Doenjang* products supplemented with *L. edodes* mushroom powder

Addition ratio of mushroom powder to *Doenjang* (%)	Color	Flavor	Taste	Overall Acceptability
Control (0%)	6.32a[a]	5.40a	5.75a	5.75a
1%	6.25a	5.45a	6.10a	6.10a
2%	6.10a	5.55a	6.45a	6.20a
3%	5.85a	5.40a	6.60a	5.60a

[a]Significantly different by paired Duncan's test at $p<0.05$
L. edodes mushroom was irradiated under UV as described before fermentation for 30 min

Table 4. Proximate analysis of *Doenjang* fortified with *L. edodes* as vitamin D sources

Sample	Alcohol (%)	Amino-nitrogen (mg%)	Moisture (%)	Soluble carbohydrate (%)
Control	0.53±0.14	514±35	55.8±1.2	0.98±0.03
Supplemented (2%)	0.52±0.17	587±28	57.3±0.7	1.02±0.04

L. edodes mushroom was irradiated under UV as described before fermentation for 30 min

Table 5. Vitamin D content (per 100 g dry weight) in traditional *Doenjang* fortified with UV-irradiated or solar-irradiated mushroom powder

Vitamin D	Soy bean	Fortified *Doenjang*		
		Control (non added)	Solar irradiated	UV irradiated
Ergocalciferol (μg)	109±8	255±13	259±14	347±26
Ergosterol (mg)	0.7±0.1	6.0±0.4	15.6±0.3	17.5±3
Total vitamin D_2 (mg)	0.8	6.3	15.8	17.9

UV-irradiated or solar-irradiated *L. edodes* mushroom powder was mixed with soybeans by 2% (dry weight/fresh weight) before fermentation.

soybean as a raw material contained ergocalciferol at a concentration of 109 μg/100 g dry weight and ergosterol at levels of 0.7 mg/100 g dry weight. In case of non-supplemented (control) *Doenjang*, these values increased to 225 μg/100 g for ergocalciferol and 6 mg per 100 g dry weight for ergosterol during fermentation. The reason was considered that the fungi and yeast such as *Aspergillus oryzae* and *Saccharomyces rouxii* synthesize vitamin D in the aging process of *Doenjang*. In case of UV-irradiated *L. edodes* supplemented *Doenjang*, these values changed to 347 μg and 17.5 mg per same weight of control. About 5 g (dry weight) of irradiated *L. edodes* were contained in a hundred grams of *Doenjang* (dry weight). This value is equivalent to approximately 75 μg/5 mg of ergocalciferol and 30 μg/4 mg of ergosterol, according to the data seen shown in Figure 1. Hence, ergocalciferol still remained at initial levels in *Doenjang*, while ergosterol contents were increased by fermentation for 3 months. This indicates that ergocalciferol was not used for population of microorganisms, while ergosterol may be dependent on microbial growth. To understand the effect of ergosterol on microbial growth in *Doenjag* fermentation, further investigation is needed.

Vitamin D is an essential compound for maintaining human health so people have to obtain it from foods or by exposing the skin to sunlight. UV irradiation was able to increase the ergocalciferol level of *L. edodes* (Figure 1) and the vitamin D content of *Doenjang* was increased by supplementation with irradiated *L. edodes* (Table 5). We can then conclude that *L. edodes*-supplemented *Doenjang* can be used as a vitamin D-fortified food.

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