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ORIGINAL ARTICLE

Changes of Isoflavone Profiles in *Cheonggukjang* with *Lentinus edodes*

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Abstract *Cheonggukjang*, a traditional soy food, was prepared with addition of *Lentinus edodes* powder at 0, 5, and 10% (w/w), and the distribution of isoflavones were analyzed by high performance liquid chromatography during fermentation for 48 h. β-Glucosidase activity and changes in succinyl genistin and succinyl daidzin were monitored. β-Glucosidase activity in *cheonggukjang* with 5 and 10% (w/w) *L. edodes* powder were significantly higher than those in control samples (p <0.05). Total isoflavones in 48-h fermented *cheonggukjang* with 0, 5, and 10% (w/w) *L. edodes* powder were 4.88, 4.26, and 3.99 μmole/g, respectively. Aglycones of isoflavones in *cheonggukjang* with 5 and 10% (w/w) *L. edodes* powder were 27.61 and 24.76% for 24 h and 28.2 and 38.74% for 48 h, whereas those in control samples were 5.50 and 21.11% for 24 and 48 h, respectively. Succinyl daidzin and succinyl genistin in *L. edodes* powder-added *cheonggukjang* were significantly lower than those of control samples (p <0.05), implying that β-glucosidase activity from *L. edodes* negatively affected the formation of succinyl derivatives.

Keywords *cheonggukjang* · β-glucosidase activity · isoflavones · *Lentinus edodes* powder

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Introduction

Cheonggukjang is one of traditional foods made of cooked and fermented soybeans mainly with *Bacillus* species for a relatively short period. Some health beneficial functionalities in c*heonggukjang* have been reported in the literature including antioxidant activity (Lee et al., 2005; Choi et al., 2008), tyrosinase-inhibiting activity (Choi et al., 2008), lowering effects of blood cholesterol level (Kim et al., 2010), and fibrinolytic activity (Joo and Park, 2010).

Isoflavones are one of major phytochemicals found in soybeans, and epidemiological health effects from the consumption of soy foods may be correlated with the isoflavones due to their structural similarity to the female hormone, estrogen (Larkin et al., 2008). Isoflavones in soy foods are composed of three aglycones such as genistein, daidzein, and glycitein and their three chemical derivatives including β-glucosides, 6"-*O*-acetyl-β-glucosides, and 6"-*O*-malonyl-β-glucosides (Lee et al., 2004). In raw soybeans, about 4.3–11.7 μg/g of total isoflavones are observed as well as 70–80% of 6"-*O*-malonyl-β-glucosides, 5% of 6"-*O*-acetyl-β-glucosides, 25% of β-glucosides, and less than 2% of aglycones are commonly detected (Lee et al., 2004). In particular, aglycones are reported to be absorbed faster than their corresponding β-glucoside derivatives in gastrointestinal organs and possess higher bioavailability (Izumi et al., 2000). Therefore, the increase of aglycones is one of major objectives for the isoflavone modification in soy foods. Chemical processes such as acid hydrolysis and biological process using microorganisms and natural products with high β-glucosidase activity have been attempted to convert isoflavone glucosides into aglycones (Shimoni, 2004; Uzzan and Labuza, 2004; Park et al., 2009). Lactic acid bacteria with high β-glucosidase activity were used to increase the content of isoflavone aglycones in soymilk (Pyo et al., 2005; Park et al., 2009). Almond powder has been used to increase isoflavone aglycones in soybread (Zhang et al., 2004).

Lentinus edodes (Pyogo) is one of frequently consumed

mushrooms in Korea and is the third most widely produced mushroom in the world (Zheng and Shetty, 2000). Health beneficial effects including antioxidative, antifungal, antiviral, and antitumor effects have been reported on this mushroom (Watanabe et al., 2003). Enzymes possessing strong β-glucosidase activity have been found in *L. edodes* (Shin, 2006). Changes in physiological and microbial properties of *deonjang* containing *L. edodes* were monitored (Rhee et al., 2000). These studies suggest utilization of the high β-glucosidase activity in *L. edodes* is necessary to modify the isoflavone profiles in soy-based foods.

Numerous attempts have been made to modify flavor and increase bioavailability of *cheonggukjang* (Shon et al., 2002; Lee et al., 2007; Choi et al., 2010). Green tea and mugwort (Lee et al., 2007), kiwi and radish (Shon et al., 2002), and red ginseng (Choi et al., 2010) are some examples of natural components used to modify attributes of *cheonggukjang*. However, to the best of our knowledge, *cheonggukjang* with *L. edodes* to modify the profiles of isoflavones has not been reported in the literature.

The objective of this study was to modify the profiles of isoflavones in *cheonggukjang* using *L. edodes*. In addition, changes in new isoflavone metabolites such as succinyl daidzin and succinyl genistin were monitored during fermentation of *cheonggukjang* containing *L. edodes* powder.

Materials and Methods

Materials. White soybeans (*Glycine max*) and *L. edodes* were purchased from a local grocery store (Seoul, Korea). Twelve isoflavones were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *Bacillus subtilis* MYCO10001 was donated by MYCO (Kyungju, Korea). HPLC-grade methanol, acetonitrile, HCl, and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ). *p*-Nitrophenol-β-D-glucopyranoside (*p*NPG), *p*-nitrophenol (*p*NP), and formononetin were purchased from Sigma Aldrich Co. (St. Louis, MO).

Preparation of *Cheonggukjang*. Based on the previous report of Yang et al. (2006), *cheonggukjang* was prepared using 500 g of washed and soaked soybeans. Soybeans were autoclaved at 121°C for 8 min (Sejong, Korea) and were inoculated with 10% (w/w) *B. subtilis* (2.7×10^7 CFU/mL). Raw *L. edodes* was ground into powder form and added to the samples to make 0, 5, and 10% (w/w) mixtures. Samples without *L. edodes* were used as control. All samples were fermented at 38°C in an incubator (KSI-220, Kukje Engineering Co., Goyang-si, Korea) and analyzed at 0, 24, and 48 h. Prepared *cheonggukjang* was stored in a −70°C refrigerator (Ilshin Lab., Yangju-si, Korea) for further analysis.

β-Glucosidase activity. The 0.1 g of *cheonggukjang* was ground and mixed with 15 mL distilled water, vortex-mixed for 1 min, and then centrifuged at 8,832 × g at 4°C for 20 min using the refrigerated centrifuge (Hanil, Incheon, Korea). The supernatant of 0.1 mL was mixed with 0.1 mL *p*-nitrophenol-β-D-glucopyranoside (pH 4.6, 9 mM) and 0.8 mL of sodium acetate buffer (pH 4.6, 0.1 M). After the mixture was shaken for 30 min, 1 mL of cold sodium carbonate (pH 9, 0.25 M) was added, and absorbance of the mixture was measured at 400 nm using a UV-spectrophotometer (UV-2101PC, Shimadzu, Tokyo, Japan). One unit (U) of β-glucosidase activity was defined as enzymes that produced 1 μmol of *p*NG from *p*NPG per min under the above assay condition (Yang et al., 2006).

Isoflavone analysis. One gram of sample was ground and mixed with solvent mixture of 7 mL acetonitrile, 2 mL of 100 mmol/L HCl, and 3 mL deionized water. An internal standard, formononetin was added to the mixture, which was vortex-mixed for 2 h and centrifuged at 2,208 × g for 10 min using a refrigerated centrifuge (Hanil). One milliliter of supernatant was dried under nitrogen gas flow.

A high performance liquid chromatograph (HPLC) with an ultraviolet detector (Hitachi Co., Tokyo, Japan) was used to analyze isoflavones according to the method of Lee et al. (2009). A Novapak C_{18} reversed-phase HPLC column (150 mm × 3.9 mm I.D., 4 μm) with a Novapak C_{18} guard column and a 0.5-μm pre-column filter from Vydac (Hesperia, CA) was the stationary phase, and mixture of 1% (v/v) acetic acid in water (solvent A) and 100% acetonitrile (solvent B) was the mobile phase. Flow rate of mobile phase was 0.6 mL/min with gradient: 85% solvent A from 0 to 5 min, 65% from 5 to 44 min, 85% from 44 to 45 min, and 85% for 5 min. Dried sample was dissolved in 1 mL methanol and filtered using a 0.2-mm syringe filter (Alltech Associates Inc., Deerfield, IL). Injection volume was 10 μL, and isoflavones were detected at 254 nm. Peaks in HPLC chromatograms were identified according to the retention times of standard compounds and quantified according to the previous reports (Lee and Lee, 2009). The amounts of new isoflavone metabolites were determined using peak response of a UV detector.

Statistical analysis. The data of isoflavones and β-glucosidase activity were analyzed statistically by ANOVA and Duncan's multiple range test using SPSS software program (SPSS Inc., Chicago, IL). A p value <0.05 was considered significant.

Results and Discussion

β-Glucosidase activity. Changes of β-glucosidase activity in *cheonggukjang* containing *L. edodes* powder are shown in Fig. 1. β-Glucosidase activity in samples containing 0, 5, 10% (w/w) *L. edodes* powder at 0 h were 0.031, 0.164, and 0.291 U/g, respectively. Preliminary study showed that raw soybeans and *L. edodes* possessed 3.5 and 19.0 U/g of β-glucosidase activity, respectively, with *L. edodes* showing 5.4 times higher β-glucosidase activity than raw soybeans.

β-Glucosidase activity in cooked soybeans is less than 1% of those in raw soybeans, which is due to the thermal inactivation of enzymes during autoclaving process (Shimoni, 2004; Uzzan and Labuza, 2004; Yang et al., 2006). As fermentation time increased to 48 h, β-glucosidase activity of control *cheonggukjang* without

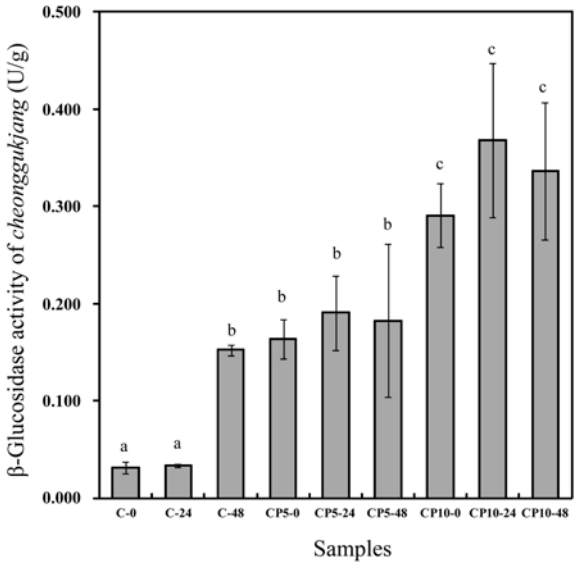

Fig. 1 β-Glucosidase activity of *cheonggukjang* added with *L. edodes* powder. Different letters are significant at $p<0.05$. Abbreviations are listed in Table 1.

addition of *L. edodes* powder increased significantly ($p<0.05$). However, β-glucosidase activities in samples containing *L. edodes* powder were not significantly different during 48 h fermentation ($p>0.05$). Considering the increases of β-glucosidase activity in control samples during fermentation, the β-glucosidase from *L. edodes* may not be able to retain its activity for 48 h.

Profiles of isoflavones. Distribution of isoflavones in *cheonggukjang* containing *L. edodes* powder during fermentation is shown in Table 1. All twelve isoflavones and two new metabolites were clearly separated in *cheonggukjang* (data not shown). Total isoflavone contents in c*heonggukjang* with 0, 5, 10% *L. edodes* powder were 5.33, 4.66, and 4.03 μmole/g, respectively. Samples containing *L. edodes* powder had lower total isoflavones due to the decrease of soybean content. As fermentation time increased to 48 h, total isoflavones in c*heonggukjang* decreased although consistent patterns were not observed.

Relative percentages of chemical forms of isoflavones are shown in Table 2. In control samples, relative percentages of aglycones, β-glucosides, acetyl-β-glucosides, and malonyl-β-glucosides were 4.09, 31.92, 5.69, and 58.30%, respectively. According to preliminary studies, the relative percentages of isoflavones in raw soybeans were 2% aglycones, 18% β-glucosides, 5% acetyl-β-glucosides, and 75% malonyl-β-glucosides. Autoclaving treatment can provide 121°C thermal energy and high moisture to raw soybeans, which decrease the content of malonyl-β-glucosides and increases that of β-glucosides. Intraconversion of isoflavones during cooking process depends on the temperature and moisture content (Shimoni, 2004; Uzzan and Labuza, 2004). Processing with high temperature with low moisture content can decrease the content of malonyl derivatives and increase that of acetyl derivatives and β-glucosides, whereas general cooking process with high moisture content decreases malonyl derivative contents without increasing that of acetyl derivatives greatly (Shimoni, 2004; Uzzan and Labuza, 2004; Lee and Lee, 2009). Aglycone content of samples with 0, 5, and 10% *L. edodes* powder for 24 h fermentation were 5.50, 27.61, and 24.76%, respectively, whereas those for 48 h were 21.11, 28.24, and 28.74%, respectively (Table 2). Samples containing *L. edodes* powder had significantly higher aglycone content than control samples during fermentation ($p<0.05$). However, relative percentage of aglycones between 5 and 10% added samples were not significant ($p>0.05$). In the case of acetyl derivatives, significant changes were not observed in all samples ($p>0.05$). Malonyl derivatives in samples with 5 and 10% *L. edodes* powder were significantly lower compared to those in control samples for 24 h ($p<0.05$), whereas no significant changes were found in 48-h fermented samples ($p>0.05$).

Rostagno et al. (2009) reported that malonyl derivatives can be converted into acetyl derivatives, β-glucosides, and aglycones. They also showed that acetyl derivatives can be converted into β-

Table 1 Distribution of isoflavones in *cheonggukjang* with addition of *L. edodes* powder during fermentation

Samples[1)]	Concentration of isoflavone (μmole/g samples)												
	DE	DI	ADI	MDI	GE	GI	AGI	MGI	GY	GYI	AGYI	MGYI	TI
C-0	0.08a[2)]	0.65e	0.17a	1.14c	0.12a	0.92c	0.04a	1.78e	0.02a	0.14bcd	0.10ab	0.19de	5.33c
C-24	0.12a	0.51bcd	0.13a	1.13c	0.13a	0.86bc	0.04a	1.68de	0.03a	0.12abcd	0.10bc	0.21e	5.05bc
C-48	0.51d	0.55cde	0.11a	0.62a	0.41c	0.95c	0.03a	1.19bc	0.11c	0.16d	0.12c	0.11abc	4.88bc
CP5-0	0.13a	0.63de	0.13a	0.89b	0.14ab	0.87bc	0.04a	1.43cd	0.03a	0.14cd	0.08a	0.14cd	4.66bc
CP5-24	0.53d	0.44abc	0.10a	0.41a	0.51d	0.81abc	0.04a	0.94ab	0.11c	0.10ab	0.10b	0.08ab	4.17ab
CP5-48	0.59d	0.41ab	0.10a	0.42a	0.51d	0.83bc	0.03a	0.96ab	0.11c	0.10abc	0.11bc	0.09ab	4.26ab
CP10-0	0.21b	0.51bcd	0.12a	0.63a	0.20b	0.80abc	0.03a	1.15bc	0.05ab	0.14cd	0.07a	0.12bc	4.03ab
CP10-24	0.39c	0.36a	0.21a	0.44a	0.35c	0.62a	0.03a	0.68a	0.09c	0.08a	0.08a	0.06a	3.41a
CP10-48	0.55d	0.38ab	0.22a	0.56a	0.50d	0.69ab	0.03a	0.74a	0.08bc	0.09a	0.09ab	0.07a	3.99ab

[1)]C, CP5, and CP10 indicate *cheonggukjang* with addition of *L. edodes* powder at 0, 5, and 10% (w/w), respectively. Number after dash indicates incubation time in hour. DE: daidzein, DI: daidzin, ADI: acetyl daidzin, MDI: malonyl daidzin, GE: genistein, GI: genistin, AGI: acetyl genistin, MGI: malonyl genistin, GY: glycitein, GYI: glycitin, AGYI: acetyl glycitin, MGYI: malonyl glycitin, TI: sum of isoflavones.
[2)]Different letters are significant in the same column at 0.05.

Table 2 Relative content of isoflavones in *cheonggukjang* with added *L.edodes* powder during fermentation

Aglycones (%)

Reaction time (h)	*L. edodes* content (%)		
	0	5	10
0	4.09A[1)]a[2)]	6.59Ab	11.36Ac
24	5.50Aa	27.61Bc	24.76Bb
48	21.11Ba	28.24Bb	28.74Bb

β-Glucosides (%)

Reaction time (h)	*L. edodes* content (%)		
	0	5	10
0	31.92Ba	35.28Bb	35.97Bb
24	29.39Aa	32.44Ab	31.53ABb
48	34.22Ba	31.40Aa	28.60Aa

6″-*O*-Acetyl-β-glucosides (%)

Reaction time (h)	*L. edodes* content (%)		
	0	5	10
0	5.69Aa	5.28Aa	5.48Aa
24	5.42Aa	5.67Aa	8.94Aa
48	5.58Aa	5.83Aa	8.31Aa

6″-*O*-Malonyl-β-glucosides (%)

Reaction time (h)	*L. edodes* content (%)		
	0	5	10
0	58.30Bc	52.84Bb	47.19Ba
24	59.68Bb	34.29Aa	34.77Aa
48	39.09Aa	34.53Aa	34.35Aa

[1)]Different capital letters are significant in the same column at $p < 0.05$.
[2)]Different small letters are significant in the same row at $p < 0.05$.

glucosides and aglycones, and β-glucosides can change into aglycones. Decreases of malonyl derivatives and increases of β-glucosides were linear with 0.99 of R^2 in soaked soybeans under a 100-dry oven (Lee and Lee, 2009). Profile changes of isoflavones in *cheonggukjang* with added *L. edodes* showed more complex patterns than un-fermented soy foods, which may be due to the presence of related enzymes from microorganisms and added natural components.

Succinyl isoflavone derivatives. Changes in peak areas of succinyl daidzin and succinyl genistin are shown in Fig. 2. Succinyl daidzein and succinyl genistin are isoflavone metabolites found in fermented soy foods with *Bacillus*, and these compounds have been reported in *natto* and *cheonggukjang* (Toda et al., 1999; Yang et al., 2008), but were not observed in non-fermented samples. As fermentation time increased to 48 h, peaks of succinyl daidzin and succinyl genistin were formed and increased. Samples with 0, 5, and 10% *L. edodes* powder were 3.24×10^6, 2.62×10^6, and 1.08×10^6 mAU, whereas those of succinyl genistin were 4.42×10^6, 3.09×10^6, and 9.76×10^5 mAU, respectively. Control samples had higher succinyl derivatives of isoflavones than samples with *L. edodes* powder ($p < 0.05$). Park et al. (2010)

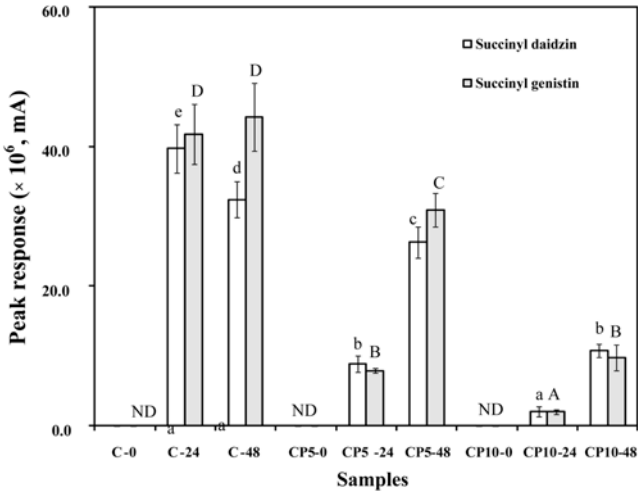

Fig. 2 Changes of succinyl daidzin and succinyl genistin in *cheonggukjang* added with *L. edodes* powder. Different capital and small letters are significant for succinyl genistin and succinyl daidzin at $p < 0.05$, respectively. ND: not detected. Abbreviations are listed in Table 1.

reported that succinyl daidzin and succinyl genistin may come from β-glucoside forms of daidzin and genistin, respectively. Therefore, aglycones and succinyl derivatives share the same precursors. Addition of β-glucosidase activity from *L. edodes* accelerates the rate of conversion from β-glucosides into aglycones and less amount of β-glucosides would remain as the precursors for succinyl derivatives. Succinyl isoflavones were not detected in Deonjang (Yang et al., 2008). Detection of succinyl derivatives of isoflavones can be a reliable indicator to discriminate authentic *cheonggukjang* from non-fermented soy foods or from fermented foods without using Bacillus species.

In conclusion, *cheonggukjang* with addition of *L. edodes* powder had significantly higher content of aglycones compared to control *cheonggukjang* without *L. edodes*. However, the increases of aglycones in *cheonggukjang* with *L. edodes* powder were not high enough considering high β-glucosidase activity from *L. edodes*. Although precursors for aglycones remained in high content, the relatively low conversion rates from β-glucosides into the corresponding aglycones may come from low enzyme stability of *L. edodes* during fermentation, which can be supported by the results of activity in Fig. 1. Addition of natural sources is one of the practical approaches to modify the isoflavone profiles in fermented soy foods such as *cheonggukjang*.

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