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RESEARCH NOTE

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Isolation of a Fermenting Microorganism Involved in Formation of *ortho*-Dihydroxyisoflavones in *Doenjang* (Korean Fermented Soybean Paste)

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Abstract A fermenting microorganism involved in formation of *ortho*-dihydroxyisoflavones (ODIs) during aging of *doenjang* (Korean fermented soybean paste) has been investigated. Microorganisms in ODI-containing *doenjang* were isolated by cultivating on yeast mold (YM) agar medium containing 0-7% NaCl. ODI formation of the isolated strains was examined by gas chromatography/mass spectrometry (GC/MS) analysis after cultivation in modified YM broth or soybean extract medium. An ODI-producing microbe was identified as *Bacillus subtilis* HS-1 based on 16S rRNA gene sequence analysis. The strain has produced 8-hydroxydaidzein as a major product during growth in the modified YM broth or soybean extract medium. Therefore, it was concluded that one of the microorganisms involved in the formation of ODIs in *doenjang* was *B. subtilis* HS-1.

Key words: *ortho*-dihydroxyisoflavone, 8-hydroxydaidzein, *Bacillus subtilis*, *doenjang*, fermentation

Introduction

Doenjang is a traditional Korean soybean paste, which is produced via fermentation by diverse microorganisms including fungi and bacilli (1-3). A number of studies showed that *doenjang* contained a variety of bioactive compounds such as functional peptides and isoflavones (4-6).

Daidzein and genistein, two most abundant isoflavones in *doenjang*, were reported to act as antioxidants, free radical scavengers, metal chelators, and antibacterial agents (6,7). Moreover, the isoflavones can be converted into *ortho*-dihydroxyisoflavones (ODIs) by microorganisms (8), which became a growing scientific interest due to their medicinal and chemo-preventive activities. Antioxidant activity of ODIs has been illustrated with oxygen radical absorbance capacity assay and oxidation of low density lipoproteins (LDL) (9). The ODIs also showed high diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity, which was stronger than their corresponding isoflavones daidzein and genistein (10,11). Moreover, antiproliferative activity of ODIs with respect to human promyelocytic leukemia cells (HL-60) was much higher than that of the corresponding daidzein and genistein (10).

The formation mechanism of ODIs was investigated with *Aspergillus saitoi*, which has shown hydroxylation activity of daidzein and genistein to 8-hydroxydaidzein and 8-hydroxygenistein in *miso*, a Japanese soybean paste (12). The ODIs were postulated to be formed by hydroxylases produced during sporulation. A more specific data was obtained from cytochrome P450 oxygenases originated from *Bacillus subtilis* 168, which showed activity for hydroxylation of daidzein to 3'-hydroxydaidzein (13).

The objectives of this study were to isolate and identify a new microorganism, which can produce ODIs during fermentation of soybean. We could illustrate for the first time that a *B. subtilis* strain, originated from *doenjang*, was able to produce 8-hydroxydaidzein as a major ODI product in the presence of daidzein.

Materials and Methods

Chemicals and culture media Daidzein, 3'-hydroxydaidzein and 6-hydroxydaidzein were purchased from Sigma-Aldrich (St. Louis, MO, USA). 8-Hydroxydaidzein was obtained from Amore-Pacific Co. (Yongin, Korea). *N,O-Bis*(trimethylsily)trifluoroacetamide (BSTFA) were purchased from Fluka (Buchs, Switzerland). The YM and MRS media were purchased from BD Difco (Franklin Lakes, NJ, USA). Soybean was obtained from a local market in Seoul, Korea.

Isolation and cultivation of microorganisms Fermenting microorganisms were isolated from *doenjang*, which has been shown to contain large amount of *ortho*-dihydroxy-isoflavones (ODIs) (14). The microorganisms were isolated by cultivating on YM or MRS agar medium containing 0-7% NaCl at 30°C.

Cultivation of the strains isolated from ODI-containing *doenjang* was carried out in modified YM or soybean extract medium at a shaking incubator (Jeio Tech Co., Daejeon, Korea) (200 rpm and 30°C). The modified YM medium consisted of 3 g/L of yeast extract, 3 g/L of malt extract, and 5 g/L of peptone. The soybean extract medium was prepared as has been reported in an earlier study (12);

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whole soybeans (400 g) were soaked overnight in 1.6 L of water at room temperature. The mixture was heated at 105°C for 60 min and then filtrated through gauze. The filtrate was centrifuged to remove the small particles. Aliquots (19.5 mL) of the soybean extract in 100-mL Erlenmeyer flasks were autoclaved at 121°C for 15 min. When the modified YM broth was used as culture medium, daidzein was added to a final concentration of 0.1 mM at the exponential growth phase.

Analysis of isoflavones Isoflavones and their hydroxy forms were analyzed by gas chromatography/mass-spectrometry (GC/MS) as has been reported in an earlier study (13); the reaction metabolites were extracted with ethylacetate (Junsei, Tokyo, Japan). The supernatant was evaporated in a centrifugal vacuum concentrator (BioTron, Bucheon, Korea) and derivatized with BSTFA for 20 min at 60°C. A Hewlett-Packard 6890 GC-5975B mass selective detector (MS) (Agilent Technology, Palo Alto, CA, USA) equipped with a HP-5ms column (30 m×0.25 mm i.d., 0.25 μm film thickness) was used. Oven temperature programming was a linear temperature gradient starting 60°C for 1 min, increasing to 250°C at the rate of 30°C/min, holding at 250°C for 10 min, increasing to 275°C at the rate of 1°C/min, and holding for 3 min. The injector port temperature was 100°C. Scan spectra was 100-550 *m/z* and mass spectra was obtained by electron impact ionization at 70 eV. The selected ion mode (SIM) was used for the detection of daidzein and the ODIs. The ODI peaks were identified based on GC chromatogram and mass spectrum of standard molecules.

Results and Discussion

Screening of fermenting microbes in *doenjang* The ODI content of *doenjang* has been shown to increase significantly over aging times. For instance, ODI content of *doenjang* increased from below 10 ppm to over 500 ppm during aging for 5 years (14). This indicates that ODI had been produced during aging of *doenjang*. In general, salt-tolerant microorganisms such as *Zygosaccharomyces rouxii*, *Candida versatilis*, and *Tetragenococcus halophilus* were reported to involve in aging of soy sauce and soybean pastes (15,16). Therefore, YM and MRS agar medium containing 0-7% NaCl was used to isolate the fermenting microbes such as salt-tolerant yeasts and lactic acid bacteria from ODI-containing *doenjang*.

A number of colonies (approximately 9.2×10^4 CFU/mg *doenjang*) came out from YM agar medium containing 7% NaCl 2 days after incubation at 30°C, but few from MRS agar medium containing 7% NaCl. Most colonies on the YM agar medium were white-colored but became transparant and slimy during further incubation.

Identification of an ODI forming microorganim ODI formation ability of the colonies isolated from ODI-containging *doenjang* was examined during cultivation in modified YM broth, which composition was identical to that of YM broth except for glucose concentration. Glucose was excluded because it might inhibit expression of cytochrome P450 oxygenases, which were reported to catalyze hydroxylation of daidzein in microorganisms (12,13). After adding daidzein to a concentration of 0.1 mM in the culture broth at the optical density (OD) of around 0.5 and further cultivation for 24 hr, the isoflavone metabolites were extracted with ethylacetate and subject to GC/MS analysis.

Table 1. *ortho*-Dihydroxyisoflavones (ODI) formation of strains isolated from ODI-containing *doenjang*

Strains	6-Hydroxydaidzein	8-Hydroxydaidzein	3'-Hydroxydaidzein
11061	-[1)]	++	+
11064	-	+	-
11066	-	-	+
05151	-	+	-
05152	-	-	+
06013	+	-	-
06014	-	-	+

[1)]+, detected (++: strong signal detected); -, not detected.

ODI formation ability of 7 strains isolated from ODI-containing *doenjang* was presented in Table 1. The ODIs such as 8-hydroxydaidzein, 6-hydroxydaidzein, and/or 3'-hydroxydaidzein were detected with GC/MS analysis, suggesting that the fermenting microbes were involved in formation of ODIs in *doenjang*.

Growth kinetics and ODI formation of a strain (11061 in Table 1) were investigated in more detail. When the strain was cultivated in the modified YM broth containing 7% NaCl under aerobic conditions at 30°C, linear cell growth was observed, which was followed by exponential growth just after initiation of the biotransformation (t=12.5 hr) (Fig. 1A). Cell growth entered the stationary phase 12 hr after biotransformation. GC/MS analysis of the sample taken at t=25 hr showed that TMS-derivatives of daidzein and 8-hydroxydaidzein were detected at the retention time of 33.6 and 40.3 min, respectively (Fig. 1B).

The strain, which showed ODI formation during the fermentation, was identified by 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of the strain showed over 99% identity with that of *B. subtilis* and thereby the strain was designated as *B. subtilis* HS-1.

Salt-tolerant *B. subtilis* strains were reported to involve in fermentation of *doenjang* (3). *B. subtilis* 168 was shown to posess a cytochrome P450 oxygenase, which was able to catalyze *ortho*-hydroxylation of daidzein (13). Based on these results, we assumed that the production of 8-hydroxydaidzein during fermentation was driven by a P450 oxygenase expressed in a salt-tolerant *B. subtilis* HS-1.

Formation of ODI in a soybean extract medium The ultimate goal of the study was to produce fermented soybean products cotaining high concentration of ODIs. Thereby, ODI formation ability of *B. subtilis* HS-1 was investigated during fermentation of soybean extract. Two different soybean extract media were used; one had NaCl supplemented and the other had no NaCl supplementation, representing *doenjang* and *cheonggukjang* (Korean fermented soybean paste made without addition of NaCl), respectively.

When soybean extract was fermented with *B. subtilis*

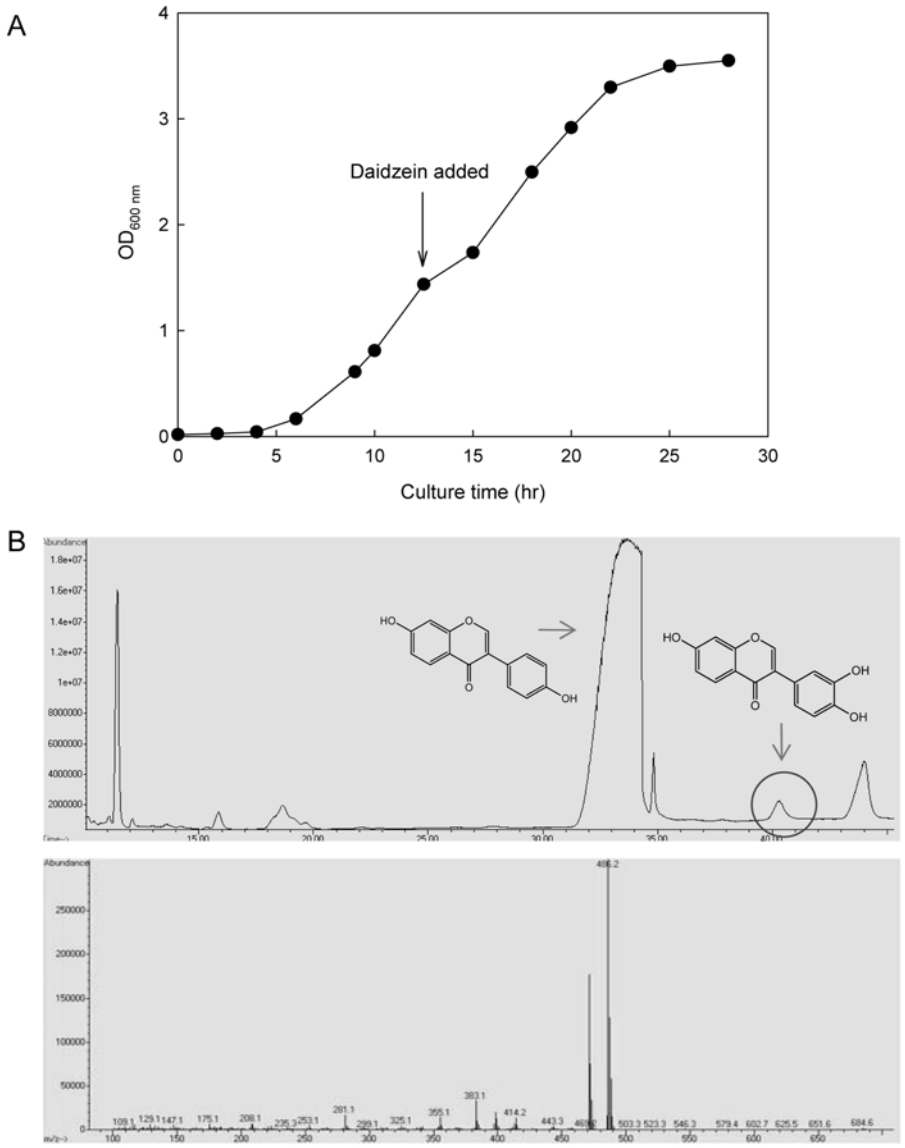

Fig. 1. Growth curve and metabolite analysis. (A) Growth curve of *B. subtilis* HS-1 in the modified YM broth containing 7% NaCl, (B) result of metabolite analysis (GC chromatogram and mass spectra of ODI) by GC/MS.

HS-1 in a shaking incubator (200 rpm and 30°C), cells grew to higher density as compared in the modified YM medium which was shown in Fig. 1A (Fig. 2A). The cell growth rate was markedly dependent on NaCl concentration; cells entered the stationary growth phase at around t=30 hr in the medium without NaCl supplementation, which was 40 hr earlier than in the medium containg 7% NaCl. Interestingly, the fermentation period of soybean extract, in which NaCl was not added, was similar to that of solid-state fermentation or *cheoggukjang*.

After cell growth entered the stationary phase in soybean extract medium with NaCl supplemented (t=93 hr), the isoflavone metabolites were extracted with ethylacetate and subject to GC/MS analysis. The GC/MS analysis showed that TMS-derivatives of daidzein and 8-hydroxydaidzein were detected at the retention time of 31.7 and 41.1 min, respectively (Fig. 2B). Almost identical result was observed in the fermentation, which was conducted without NaCl supplementation (data not shown). These results indicated that NaCl concentration had no influence on ODI formation activity.

The formation of ODIs in soybean extract medium with or without supplementation of NaCl indicated that ODIs could be produced during fermentation of *doenjang* as well as *cheonggukjang* with *B. subtilis*.

The *B. subtilis* HS-1 strain produced 8-hydroxydaidzein as a major ODI product from daidzein. This was similar to *A. saitoi*, which was shown to have hydroxylation activity of daidzein to 8-hydroxydaidzein during fermentation of soybean (12), but differed from *B. subtilis* 168, which was reported to have a P450 oxygenase capable of converting daidzein to 3'-hydroxydaidzein (13). The daidzein hydroxylation activity of the microorganisms reported so far seemed to be rather low; the highest concentration of ODI (e.g., 8-hydroxyisoflvone) in the cultivation broth

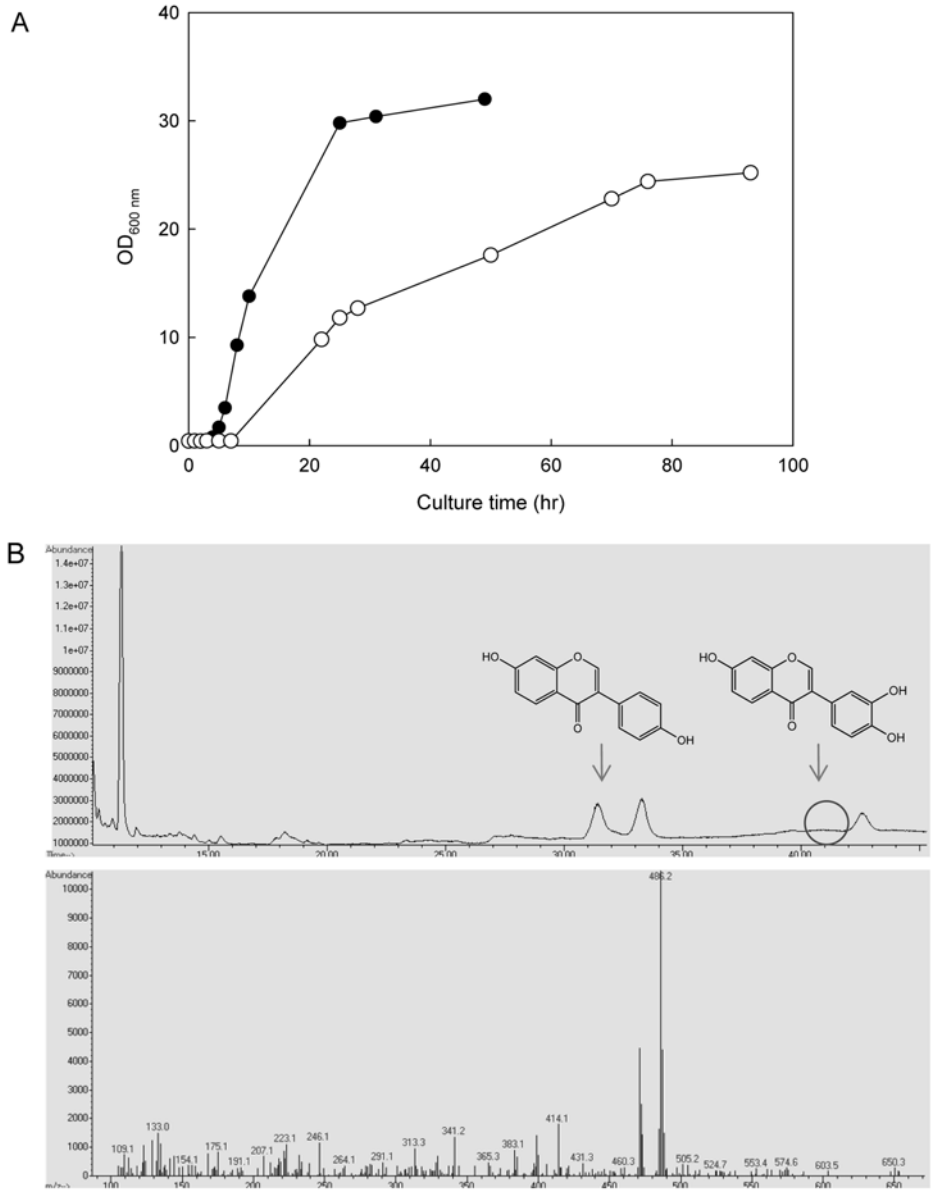

Fig. 2. Growth curve and metabolite analysis. (A) Growth curve of *B. subtilis* HS-1 in soybean extract medium containing 7% NaCl (open symbol) or without supplementation of NaCl (closed symbol), (B) result of metabolite analysis (GC chromatogram and mass spectra of ODI) by GC/MS.

remained below 0.1 mM.

Abundance of the ODI peaks on GC/MS chromatograms indicated that concentration of 8-hydroxydaidzein in the soybean extract medium was significantly lower than in the modified YM medium (Fig. 1 and 2). The low concentration of 8-hydroxydaidzein even under high cell density in the soybean medium may be due to a number of factors including low concentration of daidzein in the soybean medium and probably low expression level of P450 oxygenases, which were reported to catalyze hydroxylation of daidzein (12). The factors to influence expression level of the P450 oxygenases remained to be investigated.

This study illustrated that one of the microorganisms involved in the production of ODIs in *doenjang* was *B. subtilis* HS-1. The strain produced 8-hydroxydaidzein as a major ODI product during fermentations. We will investigate in future the factors to influece expression level of the P450 oxygenases, which were assumed to involve in hydroxylation of isoflavones, in order to improve isoflavone hydroxylation activity of *B. subtilis* HS-1.

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References

1. Lee SS. *Meju* fermentation for a raw material of Korean traditional soy products. Korean J. Mycol. 23: 161-175 (1995)
2. Yoo S-K, Cho W-H, Kang S-M, Lee S-H. Isolation and identification of microorganisms in Korean traditional soybean paste and soybean sauce. Korean J. Appl. Microbiol. Biotechnol. 27: 113-117 (1999)
3. Chang M, Chang HC. Characteristics of bacterial-*koji* and *doenjang* (soybean paste) made by using *Bacillus subtilis* DJI. Korean J. Microbiol. Biotechnol. 35: 325-333 (2007)
4. Yu B, Lu ZX, Bie XM, Lu FX, Huang XQ. Scavenging and anti-fatigue activity of fermented defatted soybean peptides. Eur. Food Res. Tech. 226: 415-421 (2008)
5. Fujita T, Funako T, Hayashi H. 8-Hydroxydaidzein, an aldose reductase inhibitor from okara fermented with *Aspergillus* sp. HK-388. Biosci. Biotech. Bioch. 68: 1588-1590 (2004)
6. Dixon RA. Phytoestrogens. Annu. Rev. Plant Biol. 55: 225-261 (2004)
7. Foti P, Erba D, Riso P, Spadafranca A, Criscuoli F, Testolin G. Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. Arch. Biochem. Biophys. 33: 421-427 (2005)
8. Lee J, Doo EH, Kwon DY, Park J-B. Functionalization of isoflavones with enzymes. Food Sci. Biotechnol. 17: 228-233 (2008)
9. Rufer CE, Kulling SE. Antioxidant activity of isoflavones and their major metabolites using different *in vitro* assays. J. Agr. Food Chem. 54: 2926-2931 (2006)
10. Hirota A, Taki S, Kawaii S, Yano M, Abe N. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging compounds from soybean *miso* and antiproliferative activity of isoflavones from soybean *miso* toward the cancer cell lines. Biosci. Biotech. Bioch. 64: 1038-1040 (2000)
11. Park JS, Park HY, Kim DH, Kim DH, Kim HK. *Ortho*-dihydroxyisoflavone derivatives from aged *doenjang* (Korean fermented soypaste) and its radical scavenging activity. Bioorg. Med. Chem. Lett. 18: 5006-5009 (2008)
12. Esaki H, Watanabe R, Onozaki H, Kawakishi S, Osawa T. Formation mechanism for potent antioxidative *O*-dihydroxyisoflavones in soybeans fermented with *Aspergillus saitoi*. Biosci. Biotech. Bioch. 63: 851-858 (1999)
13. Roh C, Choi K-Y, Pandey BP, Kim B-G. Hydroxylation of daidzein by CYP107H1 from *Bacillus subtilis* 168. J. Mol. Catal. B- Enzym. 59: 248-253 (2009)
14. Roh C. Biotransformation of *ortho*-dihydroxyisoflavones using recombinant bacterial cytochrome P450. PhD thesis, Seoul National University, Seoul, Korea (2008)
15. van der Sluis C, Tramper J, Wijffels RH. Enhancing and accelerating flavour formation by salt-tolerant yeasts in Japanese soy-sauce processes. Trends Food Sci. Tech. 12: 322-327 (2001)
16. Cho KM, Seo WT. Bacterial diversity in a Korean traditional soybean fermented foods (*doenjang* and *ganjang*) by 16S rRNA gene sequence analysis. Food Sci. Biotechnol. 16: 320-324 (2007)