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Bacterial Identification and Detection of Equol in Korean Soybean Paste

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한국 된장에서 Equol의 검출 및 미생물 동정

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Equol has beneficial effects on human health. Fermented soy products contain equol, and many microbes participate in the equol production process. This study investigated fermented Korean soybean paste, *doenjang*. Thirty seven *doenjang* samples collected from different manufacturers were examined. Equol was detected in 3 samples (D2, D13, and D19) at the maximum content of 507 ng/100 g by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Fifteen microbial species were isolated and identified by 16S rRNA gene sequence analysis and by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). *Bacillus* spp, *Paenibacillus* spp, *Tetragenococcus* spp, *Stapylococcus* spp, and *Clostridium* species were the predominant bacteria in equol containing *doenjang* samples.

Keywords: Equol, LC-MS/MS, MALDI-TOF mass spectrometry, 16S rRNA gene sequence

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Introduction

Isoflavones are organic compounds found in plants including soybeans. They are referred to as phytoestrogens because they are structurally and functionally similar to estrogen, and have potentially beneficial effects on human health. Epidemiologic and experimental studies have indicated preventive effects on breast cancer, prostate cancer, cardio-vascular disease, and symptoms of osteoporosis and menopause (Adlercreutz, 2002).

Equol (4',7-dihydroxy-isoflavandiol) is a metabolite of daidzein, a major soybean isoflavone. Interest is increasing in equol due to its beneficial effects on human health (Sathyamoorthy & Wang, 1997). Equol is converted via dihydrodaidzein to *O*-desmethylangolensin (O-DMA) or equol by enzymes of intestinal bacteria (Bowey *et al.*, 2003).

The potent bioactivity of equol led to a boost of investigations in separation of the specific bacteria responsible for converting daidzein to equol. To date, several bacteria capable of producing equol have been separated from human or animal feces (Maruo *et al.*, 2008; Matthies *et al.*, 2008; Jin *et al.*, 2010). Many bacteria that convert daidzein to equol have

not been isolated from foods.

Stinky tofu, a traditional fermented soy food in Taiwan, is enriched in *S*-equol (Abiru *et al.*, 2012; Jou *et al.*, 2013). It has been assumed that equol-producing bacteria may be involved in equol production during the fermentation processes of stinky tofu.

Based on this background, we measured the equol contents in fermented Korean soybean pastes and identified bacteria using 16S rRNA gene sequence analysis and two matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), using a Bruker Microflex MS (BMS) and VITEK MS (VMS) systems (Kim *et al.* 2015).

Materials and Methods

1. Chemicals

The reference standard, *S*-equol was purchased from Cayman (Ann Arbor, MI, USA). High-pressure liquid chromatography (HPLC) grade methanol and acetonitrile (ACN) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (liquid chromatography-MS grade) and HPLC grade dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical grade quality and all solvents were HPLC grade.

2. Sample preparation and extraction

Thirty seven *doenjang* samples (D1 to D37) made using the traditional Korean process were collected from manufacturers and immediately stored at −20°C. On the day of the analysis, the sample was thawed at room temperature and approximately 1 g of sample was added to 10 mL methanol and mixed on a vortex mixer until a homogenous sample was obtained. The mixture was sonicated for 15 min and 2 mL aliquots were centrifuged at 12,000 rpm for 10 min at 4°C to precipitate proteins. Aliquots of the supernatant (1.5 mL) were individually transferred to 2.0 mL tubes and placed in a vacuum concentrator. The dried residue was reconstituted in 0.1 mL of 50% ACN by vortex mixing. After 10 min of sonication, the supernatant (0.1 mL) was extracted and loaded into a Costar Spin-X centrifuge tube filter (0.22 μm; Nylon Corning Incorporated, Corning, NY, USA) and centrifuged at 3,000 rpm for 10 min. The extract was transferred to HPLC-vials before injection in the liquid chromatography-tandem mass spectrometry (LC-MS/MS) device.

3. LC-MS/MS instrumentation and analytical conditions

Mass spectrometric detection was performed on a 3200 QTRAP® (AB Sciex, Foster City, CA, USA) using a hybrid triple quadrupole linear ion trap equipped with a TurboIonspray ion source and electrospray ionization (ESI) probe. The mass spectrophotometer was set at the negative mode. The parameters were: source temp=600°C, ion spray voltage=−4500 V, declustering potential=−30 V, entrance potential=−4.5 V, collision energy=−28 V, curtain gas=25 psi, cad gas=medium, gas1=55 psi, and gas2=55 psi The data were processed using Analyst® 1.5 version software. LC separations were performed by using a 1200 series HPLC apparatus (Agilent Technologies, Santa Clara, CA, USA). The HPLC module consisted of an Agilent 1200 series binary pump, Agilent 1200 series micro-vacuum degasser, and Agilent 1200 series autosampler. The analytes were chromatographed on a YMC Triart C18 column (2.1 mm×100 mm, S-3 μm, 12 nm; YMC Co. Ltd., Kyoto, Japan) at a flow rate of 200 mL/min with gradient elution of (0.05% formic acid and 80+20% deionized water – acetonitrile) using the gradient steps (Table 1). The injection volume was 15 μL and the autosampler was operated at room temperature.

4. Isolation

Doenjang samples were cultured on thioglycollate broth (BD, Heidelberg, Germany) for 16~24 h at 37°C. After

Table 1. HPLC pump gradient elution of the equol for each run

Step	Time (min)	Flow rate (mL/min)	%A 0.05% FA in DI	%B 0.05% FA in ACN
Initial	0	0.2	80	20
2	2	0.2	80	20
3	4	0.2	20	80
4	6	0.2	20	80
5	6.2	0.2	80	20
6	9	0.2	80	20

Abbreviation: FA, formic acid; DI, deionized water; ACN, acetonitrile.

enrichment of the samples, the cultures were grown on blood agar plates for 16~24 h in a anaerobic condition provided by CampyGen (Becton Dickinson, GasPack) for isolating the organisms.

5. MALDI-TOF VITEX MS (VMS) and BRUKER MS (BMS)

VMS and BMS target slides were prepared according to the manufacturer's instructions. The resulting slides of VMS were analyzed in a VITEX MS device (bioMerieux) using the automatic database analysis of the obtained mass spectra within MYLA software (bioMerieux) to provide isolate identification. The results were evaluated according to a colored index: green for ≥90% identity, yellow for 85~89.9% identity, and white for <85% identity. All of the identifications to the genus or species level fell into the green zone, with a score of >90% considered reliable. Scores between 85 and 90% were considered as acceptable identification. A cut-off of 90% was chosen for the VITEX MS.

Measurements of BMS were done with a microflex LT mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) using flexControl™ software. The identification was provided by a score of reliability [A log (score) value between 0.00 and 3.00 was calculated : <1.7=ID not reliable; 1.7≤ and <2.0=ID at genus level; 2.0≤=ID at species level]. A result was considered species consistent if all matches with a log score between 1.7 and 2.0 corresponded only to other species of the same genus. A genus consistent result was accepted if all log scores 1.7≤ belong to the same genus.

Bacillus subtilis ATCC 6051 and Escherichia coli ATCC 8739 were included as positive controls and matrix alone with no organism was included as a negative control in each run in both MALDI-TOF systems. Discrepancies were defined as different genus- or species- level identification obtained from the VITEX MS, Microflex Bruker MS. Whenever there was a discrepancy, the analysis was repeated by both systems to eliminate the possibility of contamination. Thereafter, any other disagreements in the identification were resolved by performing 16S rRNA gene sequencing on the discrepant isolates.

6. 16S rRNA gene sequencing

To prepare cells for DNA extraction, the cultivated strains were collected by centrifugation at 14,000 ×g for 5 min at 4°C. The resulting pellet was extracted using a QIAamp DNA minikit (Qiagen, Valencia, CA, USA). The extracted DNA was used as a PCR template for the amplification of 16S rRNA genes with the universal primer pair 5'-AGAGTTTGATC-CTGGCTCAG-3' (27F) and 5'-GGCTACCTTGTTACGACTT-3' (1492R). The 16S rRNA gene was amplified by PCR with a thermal cycler (Applied Biosystems, Foster City, CA, USA). The amplified products were purified with a QIAquick PCR purification kit (Qiagen). The PCR products were sequenced using the ABI Big Dye v3.1 Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems) using manufacturer's protocol. The samples were purified to remove excess salt and unincorporated dNTPs, denatured with HiDi-Formamide at 95°C for 3 min, and analyzed using 3730 DNA Analyzer (Applied Biosystems). The obtained sequences were compared with sequences present in the GeneBank database (National Center for Biotechnology Information Bethesda, MD, U.S.A.) using BLAST software (http://www.ncbi.nih.gov).

Results

1. Determination of equol by LC-MS/MS

Equol contents of 37 *doenjang* samples collected in this study were determined by LC-MS/MS (Table 2). As shown in Fig. 1, the chromatographic retention time of standard solution, *doenjang* sample 2 (D2), *doenjang* sample 13 (D13), *doenjang* sample 19 (D19) was 5.52 min, 5.54 min, 5.52 min, and 5.53 min, respectively. Peak of equol was detected and the equol fraction contained a single peak with a retention time corresponding to that of the standard solution.

Relatively high concentrations were detected in the three samples (350 ng/100 g in D2, 507 ng/100 g in D13, and 368 ng/100 g in D19) compared to the other samples. Equol was qualitatively determined by comparison with standard chromatogram and by doping the samples with standard reference.

2. Bacterial communities of *doenjang* samples

Microbes in D2, D13, and D19 were isolated and identified. As shown in Table 3, both BMS and VMS correctly identified 1 *Bacillus licheniformis*, 1 *Staphylococcus hominis*, 1 *Staphylococcus warneri*, 1 *Bacillus cereus*, 1 *Bacillus circulans* to the species level. However, both systems misidentified *Clostridium xylanolyticum* as *Clostridium clostridioforme* and *Clostridium aerotolerans*, respectively.

BMS misidentified 1 *Bacillus licheniformis* as *Bacillus sonorensis*, 1 *Bacillus amyloliquefaciens* as *Bacillus subtilis*, 4 *Bacillus subtilis* as *Bacillus amyloliquefaciens*, 1 *Tetragenococcus halophilus* as *Lactobacillus amylovorus* compared to the sequencing results.

VMS misidentified 1 *Paenibacillus odorifer* as *Bacillus*

Table 2. Equol contents (ng/100 g) of 37 *Doenjang* samples

Sample	Equol content	Sample	Equol content
D1	4	D19	368
D2	350	D20	10
D3	52	D21	187
D4	98	D22	128
D5	238	D23	27
D6	123	D24	248
D7	77	D25	12
D8	39	D26	11
D9	10	D27	207
D10	104	D28	80
D11	57	D29	37
D12	95	D30	29
D13	507	D31	107
D14	234	D32	44
D15	22	D33	113
D16	28	D34	69
D17	54	D35	80
D18	53	D36	46
		D37	4

Abbreviation: D, *Doenjang*.

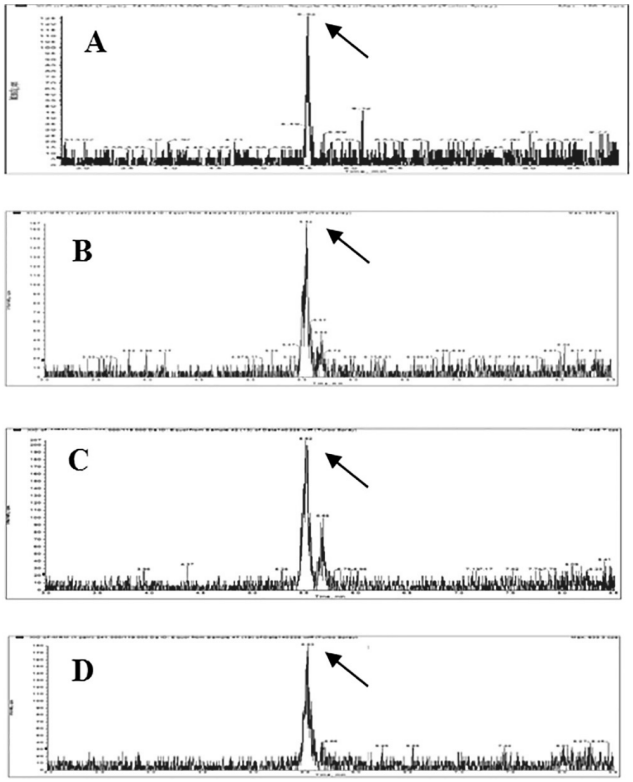

Fig. 1. Chromatographic retention time of: (A) Standard solution, (B) *Doenjang* sample 2 (D2), (C) *Doenjang* sample 13 (D13), (D) *Doenjang* sample 19 (D19). The peaks of equol are pointed by arrows.

Table 3. Bacterial identification of equol containing *doenjang* samples

MALDI-TOF MS		16S rRNA sequence results	Final identification
VITEX MS (VMS)	Bruker MS (BMS)		
Bacillus licheniformis	*Bacillus licheniformis*		*Bacillus licheniformis*
Bacillus licheniformis	*Bacillus sonorensis*	*Bacillus licheniformis*	*Bacillus licheniformis*
Bacillus licheniformis	*Paenibacillus odorifer*	*Paenibacillus odorifer*	*Paenibacillus odorifer*
Bacillus amyloliquefaciens	*Bacillus subtilis*	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*
Clostridium clostridioforme	*Clostridium aerotolerans*	*Clostridium xylanolyticum*	*Clostridium xylanolyticum*
Bacillus subtilis (4)	*Bacillus amyloliquefaciens* (4)	*Bacillus subtilis* (4)	*Bacillus subtilis* (4)
Staphylococcus hominis	*Staphylococcus hominis*		*Staphylococcus hominis*
Tetragenococcus halophilus	*Lactobacillus amylovorus*	*Tetragenococcus halophilus*	*Tetragenococcus halophilus*
Staphylococcus warneri	*Staphylococcus warneri*		*Staphylococcus warneri*
Paenibacillus peoriae	*Paenibacillus polymyxa*	*Paenibacillus polymyxa*	*Paenibacillus polymyxa*
Bacillus cereus	*Bacillus cereus*		*Bacillus cereus*
Bacillus circulans	*Bacillus circulans*		*Bacillus circulans*

Discrepant results were resolved by 16S rRNA gene sequencing.

licheniformis, 1 *Paenibacillus polymyxa* as *Paenibacillus peoriae*. *Clostridium clostridioforme* and *Clostridium aerotolerans* were not present in its database but identified by 16S rRNA sequencing as *Clostridium xylanolyticum*. Using 16S rRNA sequencing results as the reference standard, misidentified results from two different MALDI-TOF MS systems were confirmed. Their sequences showed more than 99% identity with those of the GenBank database.

Discussion

Korean soy foods are increasingly present on the worldwide market. Fermented soybean paste, *doenjang*, was registered in CODEX on July 4, 2009, and is now an internationally accepted food (Yang *et al*., 2011). It is manufactured by soaking, steaming, and fermenting soybeans in a humid closed space maintained at 30°C for specific periods. Fermentation is one of the major processes used in the production of *doenjang* and many microbes participate in fermentation process.

It has been reported that stinky tofu, a traditional and popular fermented soy food, was found to be rich in equol and many microbes participated in equol production process (Abiru *et al*., 2012). Also, several studies showed that many types of isoflavones were detected in the fermented Korean soybean paste. To the best of our knowledge, there have been no investigations up till now regards equol contents of fermented Korean soybean paste. Based on these points, we hypothesized that fermented Korean soybean paste may contain equol contents. Organisms identified in equol containing *doenjang* samples will have differences compared with dominant organisms in normal *doenjang* (i, e., *Bacillus subtilis*, *B. licheniformis*) that play important roles during fermentation. The present study assessed this hypothesis.

The present analysis revealed a maximum equol content of 507 ng per 100 g *doenjang*. Four *Bacillus subtilis*, two *Bacillus licheniformis*, and one each of *Paenibacillus odorifer*, *Bacillus amyloliquefaciens*, *Staphylococcus hominis*, *Clostridium xylanolyticum*, *Tetragenococcus halophilus*, *Staphylococcus warneri*, *Paenibacillus polymyxa*, *Bacillus cereus*, and *Bacillus circulans* were identified in equol containing *doenjang* samples. Equol content in *doenjang* samples varied. The variation may reflect a number of factors, such as differences in the fermentation environment and/or the state of raw materials.

Limitations of this study were the relatively small number of samples and microbes identified in the current methods. The analyses of the diverse microbes in *doenjang* samples was limited because of the culture-dependent methods, which only identify isolated strains growing on specific nutrient media (Yoo *et al*., 1999). Addition to foods reported to contain equol, such as dairy and egg products (Antignac *et al*., 2004), the current study shows that fermented Korean soybean paste also contains equol and demonstrates that *doenjang* can be a good dietary source of equol. Additional studies will be needed to analyze diverse microorganisms that involved in equol containing *doenjang* samples by culture-independent analysis method in many *doenjang* samples.

In conclusion, fermented Korean soybean paste is a food that contains the equol, which we detected for the first time. The results demonstrate fermented Korean soybean paste served as a good dietary source of equol and it is of great interest to conduct further studies on identification of the specific equol-producing bacteria.

요 약

에쿠올은 인간의 건강에 유익한 효과를 나타낸다. 발효된 콩 식품들은 에쿠올을 함유하고 있으며, 많은 미생물들이 에쿠올 생산 과정에 참여하는 것으로 밝혀졌다. 본 연구에서는 한국의 전통 발효 식품인 된장에 대해 조사하였다. 먼저 서로 다른 제조자로부터 수집 된 37개의 된장 샘플들을 대상으로 에쿠올의 농도를 측정하기 위해 LC-MS/MS를 시행하였다. 측정 결과 3개의 된장 샘플에서 에쿠올이 검출되었고, 507 ng/100 g의 농도가 가장 높게 나타났다. 에쿠올을 함유한 된장에서 15개의 미생물 종들이 16S rRNA gene sequence analysis와 2개의 MALDI-TOF MS분석법에 의해 분리, 동정되었으며 *Bacillus* spp, *Paenibacillus* spp, *Tetragenococcus* spp, *Stapylococcus* spp, and *Clostridium* species들이 가장 우세한 미생물들이었다. 이 연구결과로 한국의 전통 발효 식품인 된장에서도 에쿠올이 검출되었음을 확인하였다.

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