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Antimicrobial Compounds Profile During *Cheonggukjang* Fermentation Against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)

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***Xanthomonas oryzae* causes rice bacterial blight, which has been reported as one of the most destructive diseases of rice. Metabolites were identified through *cheonggukjang*, a traditional Korean fermented soybean product fermented by the *Bacillus* spp., to control the bacteria. HPLC, MS, and UPLC-Q-TOF-MS analyses were performed to identify metabolites responsible for antimicrobial activity. In this analysis, the *m/z* values of 253.0498, 283.0600, 269.0455, 992.6287, and 1,006.6436 were identified as daidzein, glycitein, genistein, surfactin B, and surfactin A, respectively. The levels of surfactin B and surfactin A were found to be high at 24 h (4.35 μg/ml) and 36 h (3.43 μg/ml) of fermentation, respectively.**

Keywords: Antimicrobial activity, fermentation, *cheonggukjang*, UPLC-Q-TOF-MS

Traditional soya foods are famous in most Asian countries and include soybean milk, beverages, tofu, miso, and natto. In particular, *cheonggukjang* fermented by *Bacillus* spp. is used in traditional Korean fermented soybean products [7, 11, 23]. The quality of *cheonggukjang* depends on the inoculated bacterial strains, soybean varieties, fermentation time, and the sub-ingredients ratio [15]. *Cheonggukjang* has been reported to show antimicrobial and other beneficial activities by Kim *et al*. [9] and Sugano *et al*. [20]. *Cheonggukjang* is consumed not only for its nutritional value but also for the biological beneficial effects due to fermentation [14]. Metabolite profiling of such fermented products has led to the identification of various compounds that are beneficial to human health. LC–MS- and GC–MS-based metabolic profiling of those traditional fermented products can be used to identify various biomolecules [8].

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the most destructive disease and causes bacteria blight in rice crops [1]. *Xanthomonas oryzae* pv. *oryzae* KACC 10331 was defined as a remarkable virulent Korean strain [13]. In order to control this plant disease, commercial chemicals have been used in the fields; however, worldwide use of pesticides is gradually decreasing because of their excessive toxicity and degradation properties. Thus, it is necessary to identify safe natural bioactive substances that can be used as alternatives to such pesticides.

Thus, in the present study, the antimicrobial activity of compounds synthesized during *cheonggukjang* fermentation against *Xanthomonas oryzae* was investigated. Compounds shown to inhibit rice bacterial blight were identified and analyzed by HPLC and LC–MS/MS.

Cheonggukjang (CGJ) powder was received from the Korean Food Research Institutes, inoculated with the *Bacillus licheniformis* KCCM 11053P, and fermented for 0, 12, 24, 36, 48, 60, and 72 h at 42°C. The CGJ powder (3 g) collected after fermentation was extracted with methanol / water [80:20 (v/v)]. The obtained extraction that was fractionated with ethyl acetate / water [2:1 (v/v)] adapting solvent partition was used for analysis. Preparative reverse phase high-performance liquid chromatography (RP-HPLC) equipped with a YMC-Pack Pro C18 column was used in order to purify the active compounds. A Varian 500-MS ion-trap mass spectrometer with Varian PurSuit XRs C18 column was used for compound identification. The gradient elution contained mobile phase A, which consisted of water and 0.1% formic acid (v/v), and B, which consisted of acetonitrile and 0.1% formic acid (v/v). Samples were eluted at a flow rate of 0.2 ml/min. The mass range was designated from *m/z* 100 to 1,000 under the negative mode of LC-ESI-MS. A Waters Micromass Q-TOF Premier with UPLC Acquity system and Acquity UPLC BEH C_{18} column was used for confirming the results. HPLC-grade solvents were used for the analysis and all the standards were purchased from sigma.

Xanthomonas oryzae KACC 10331 cultured in Muller–Hinton broth medium was collected and diluted 1:20 (v/v). In order to determine the minimal inhibitory concentrations

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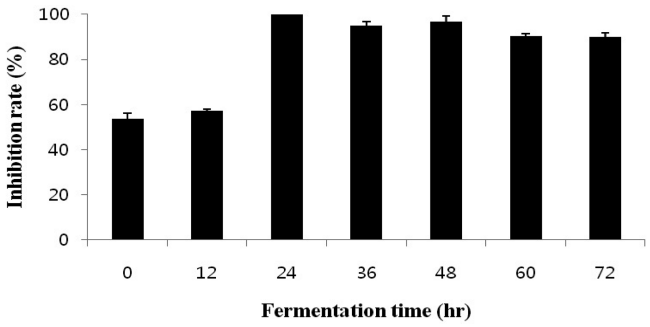

Fig. 1. Antimicrobial activity of fermented *cheonggukjang* against *Xanthomonas oryzae.*
Inhibition rates of the ethyl acetate extract of *cheonggukjang* fermented by *Bacillus licheniformis* KCCM 11053P were determined by MIC assay.

(MICs) of the active compounds, the serial dilution method was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) [4, 22] to obtain a concentration range of 0.5 to 128 ppm [3]. A GENESYS 6 UV–Vis Spectrophotometer was used to measure the OD at 600 nm.

Cheonggukjang samples fermented with *Bacillus licheniformis* KCCM 11053P for a period of 0, 12, 24, 36, 48, 60, and 72 h were extracted with 80% aqueous methanol. To evaluate the antimicrobial activity against *Xanthomonas oryzae* (*Xoo*), MIC tests were conducted with partitioned ethyl acetate extracts. The antimicrobial activity of the samples obtained at different fermentation times are presented in Fig. 1. The samples fermented for 24 to 72 h showed higher activities against *Xoo*. The activity of the samples obtained after 0 and 12 h of fermentation showed lower activity against *Xoo*.

To identify and isolate antimicrobial compounds, 24 h fermented *cheonggukjang* was selected. Preparative HPLC was performed for the separation and all the MIC values of all obtained compounds were measured. Five peaks at retention times of 27, 28, 34, 58, and 60 min showed antibacterial activity against *Xoo* (Fig. 2). To identify and quantify the compounds active against *Xoo*, LC–ESI/MS and UPLC-Q-TOF/MS analyses were performed and the results are presented in Table 1. From the ethyl acetate layer of 24 h *cheonggukjang*, the *m/z* values of the 5 compounds were at 253, 283, 269, 993, and 1,007 by LC–

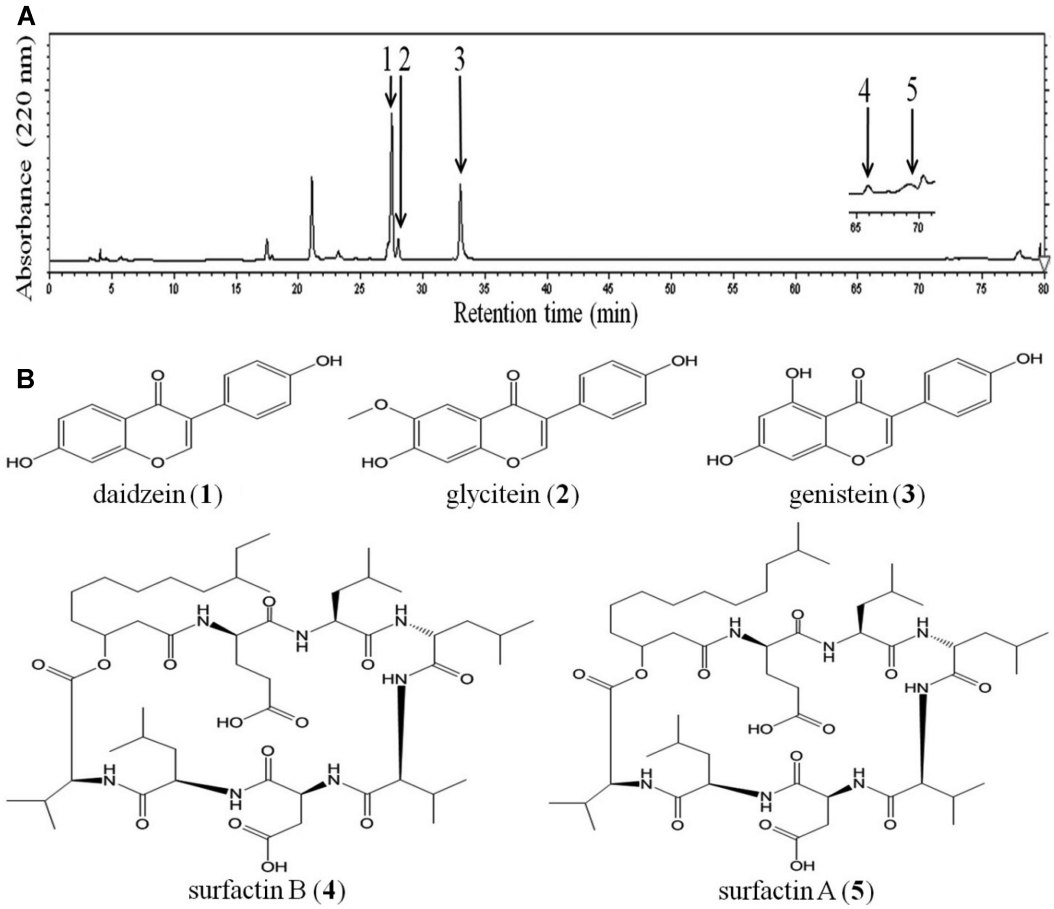

Fig. 2. HPLC chromatogram (**A**) of *cheonggukjang* fermented by *Bacillus licheniformis* KCCM 11053P for 24 h, and antibiotic compound structures (**B**).

Table. 1. LC–ESI/MS and UPLC-Q-TOF/MS based antimicrobial compound identification from fermented *cheonggukjang*.

Tentative identification	LC–ESI/MS			UPLC-Q-TOF/MS			Formula	UV λmax (nm)	Ref.
	t_R (min)	M.W	$[M-H]^-$ MS^n fragment ions (*m/z*)	Measured $[M-H]^-$	Calc. $[M-H]^-$	Error (ppm)			
Daidzein (**1**)	13.4	254	253 > 224, 195, 166	253.0498	253.0501	-1.2	$C_{15}H_{10}O_4$	260	Std., [17, 19, 20]
Glycitein (**2**)	13.5	284	283 > 268, 240, 196	283.0600	283.0606	-2.1	$C_{16}H_{12}O_5$	259	Std., [17, 19, 20]
Genistein (**3**)	15.4	270	269 > 181, 171, 155, 154	269.0455	269.0450	1.9	$C_{15}H_{10}O_5$	250	Std., [16]
Surfactin B (**4**)	29.7	994	992 > 650, 452, 339	992.6287	992.6284	0.3	$C_{50}H_{87}N_7O_{13}$	205	Std., [5, 12]
Surfactin A (**5**)	30.0	1,008	1,007 > 665, 451, 425, 339	1,006.6436	1,006.6440	-0.4	$C_{51}H_{89}N_7O_{13}$	212	Std., [5, 12]

t_R, Retention time; MW, molecular weight; Calc., calculated mass; Ref., reference; Std., Standard compound.

ESI/MS under negative mode. The high-resolution UPLC-Q-TOF/MS indicated the *m/z* values of the compounds at $[M-H]^-$ 253.0498, 283.0600, 269.0455, 992.6287, and 1,006.6436, and the candidate formulas were estimated as $C_{15}H_{10}O_4$, $C_{16}H_{12}O_5$, $C_{15}H_{10}O_5$, $C_{50}H_{87}N_7O_{13}$, and $C_{51}H_{89}N_7O_{13}$ from the *m/z* values. Based on the above data, the compounds were determined to be daidzein, glycitein, genistein, surfactin B, and surfactin A, with an error ppm of -1.2, -2.1, 1.9, 0.3, and -0.4, respectively (Table 1).

The activity was confirmed using standards of daidzein, glycitein, genistein, and surfactin. The amount of the compounds daidzein, glycitein, genistein, surfactin B, and surfactin A were quantified at different time intervals and are presented in Table 2. A gradual increase was observed with daidzein, glycitein, and genistein synthesis against *cheonggukjang* fermentation time. The surfactin B and surfactin A were found to be high after 24 h (4.35 μg/ml) and 36 h (3.43 μg/ml) of fermentation, respectively.

Cheonggukjang fermentation is a non-thermal process in which chemical changes are caused by enzymes produced by *Bacillus* sp, the main microorganism in *cheonggukjang* [2]. The *Bacillus* species typically used for this type of fermentation includes *B. subtilis*, *B. pumilus*, and *B. licheniformis*, and the distribution of isoflavonoids differs based on the strains inoculated [3, 23]. The *Bacillus* sp. produces surfactin and it shows strong activity against microorganisms [6, 10, 18]. According to Kowall *et al*. [5] and Huang *et al*. [12], the surfactin was found to be a potent biosurfactant that contains high antimicrobial activity. Our results indicated that the amount of surfactin B was high at 24 h and surfactin A was high at 36 h of fermentation, leading to remarkable antimicrobial activity.

The amount of isoflavone aglycones synthesized increases during the fermentation of soybean products. Owing to this increase, the antimicrobial activity of soya products increases as well. The antioxidant activity of genistein was demonstrated by Record *et al*. [16]. A high concentration of glycitein and daidzein served as good antimicrobial compounds produced during soy fermentation [17, 19, 20]. Our result revealed that the amount of isoflavonoid aglycone production was high at 72 h of fermentation, but the surfactin A and B content was high after 24 and 36 h of fermentation. Even though the production of isoflavonoid aglycone was high after 72 h of fermentation, the antimicrobial activity was high after 24 h because of the higher surfactin A and B concentrations. Based on our experiments, we found that the concentration of three isoflavonoid aglycones, glycitein, daidzein, and genistein, and two peptides, surfactins A and B, increased during fermentation of *cheonggukjang*.

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Table. 2. The production profile of antibiotic compounds during *cheonggukjang* fermentation time.

Fermentation time (h)	Daidzein (**1**)	Glycitein (**2**)	Genistein (**3**)	Surfactin B (**4**)	Surfactin A (**5**)
0	< 0.78	< 0.78	<0.78	< 0.78	< 0.78
12	< 0.78	< 0.78	< 0.78	< 0.78	< 0.78
24	1.54	< 0.78	0.89	4.35	1.25
36	2.30	< 0.78	1.01	4.12	3.43
48	3.13	0.93	1.92	4.07	4.66
60	3.68	1.2	2.62	4.07	4.16
72	3.78	1.47	2.89	3.29	4.28

(μg/ml)

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