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Cytotoxic, Antioxidative, and ACE Inhibiting Activities of Dolsan Leaf Mustard Juice (DLMJ) Treated with Lactic Acid Bacteria

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Abstract This study was performed to know whether there is any change of physiological activity in DLMJ which is inoculated by lactic acid bacteria. Lactic acid bacteria were isolated from Dolsan leaf mustard Kimchi (DLMK) at 20°C. In the optimum ripening period, the population of *Leuconostoc* and *Lactobacilli* in the DLMK were found to be high. The *Leuconostoc, Lactobacilli* and *Lactococci* strains were identified as *Leuconostoc mesenteroides, Leuconostoc gelidum, Weissella confusa, Lactobacillus plantarum, Lactobacillus raffinolactis, Lactococcus lactis* and *Weissella confusa* using the Biolog system. The most predominant strain which was isolated from DLMK was *Weissella confusa.* As the results of the phylogenetic analysis using 16s rDNA sequence, the *Weissella confusa* turned out to be *Weissella kimchii,* with 99.0% similarity. To investigated the change of physiological activity in DLMJ by lactic acid bacteria, 7 predominant strains inoculated to DLMJ (Dolsan Leaf Mustard Juice). The cytotoxicity was found to be under 19.55% all cases. Also, the antioxidative activity of the DLMJ treated with lactic acid bacteria was very low, which might have been due to the reduced antioxidative phytochemicals during the preparation of the sterile sample. The ACE inhibiting activity of DLMJ by inoculation with *Weissella kimchii* was shown to be the highest (94.0%). This could be that the degradation of sulfur containing materials in DLMJ by *Weissella kimchii* gave rise to ACE inhibiting activity.

Keywords: dolsan leaf mustard juice, *Weissella kimchii,* cytotoxicity, antioxidative, ACE inhibiting activity

INTRODUCTION

Leaf mustard (*Brassica juncea* Coss. var. integrifolia) is a representative of the family cruciferae (*Brassicaceae*), and has been identified as the major ingredient for Gat-Kimchi (leaf mustard Kimchi), which is known for its unique flavor and hot taste. Leaf mustard is a richest source of the known physiological compounds; glucosinolates, ascorbic acid, β-carotene, chlorophyll, dietary fiber and flavonoides [1,2]. Glucosinolates can contain at least 120 different aglycons [3], and many plant secondary metabolites are currently isolated by solvent extraction from naturally grown whole plants [4]. Of these compounds, 4-benzyl isothiocyanate, isolated from the seeds of *Moringa oleifera* and *M. stenopetala* has been identified as an active antimicrobial agent [5]. Also, the indole glucosinolates in vegetables of the genus *Brassica* have been shown to inhibit carcinogenesis in experimental animals.

Leaf mustard Kimchi (LMK) is traditionally fermented in Yoesu, Korea using various lactic acid bacteria. In the present research [6], the microbe numbers were significantly related to the physiological activity of LMK. These results suggest that lactic acid bacteria would play most important roles, not only in improving the quality, but also the physiological activity of LMK. The lactic acid bacteria in Kimchi are comprised of the genera *Lactobacillus, Leuconostoc* and *Pediococcus*, and the newly recognized *Carnobacterium*, which contribute to the flavor, color development and preservation of foods [7]. Also, the lactic acid bacteria in Kimchi show an antimicrobial activity due to the production of organic acids, hydrogen peroxide, carbon dioxide and bacteriocins [8-11]. Moreover, newly isolated and identified strains have been reported, such as *Lactobacillus kimchii* [12], *Leuconostoc kimchii* [13] and *Weissella kimchii* [14]. The genus *Weissella* was recently isolated due to the developments in DNA technology [15]. *Weissella confusa* (previously known as *Lactobacillus confusus* and originally known as *Lactobacillus coprophilus*) is present in the normal microflora of human intestines [16-18] and has anti-*Helicobacter pylori* functions [19]. The *Weissella kimchii* is the nearest phylogenetic relative of *Weissella confusa,* with a 16S rRNA similarity of 98.3% [14].

Choi *et al.* [20] has reported that cruciferous vegetables include various enzymes, such as polygalacturonase, pectinesterase, α-amylase and β-amylase, and myrosinase

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is also released when the cells in plants are damaged. And the various extracellular enzymes, such as α-amylase, β-amylase, protease, pectinesterase and polygalacturonase, produced by lactic acid bacteria from Kimchi. These results suggested that the possibility of physiological hydrolysis compounds were produced in DLMJ by lactic acid bacteria. In previous our research [21], the microorganisms in DLMK juice would play important role in the antioxidative and ACE inhibiting activity. And, also ACE inhibiting activity in DLMJ was significantly related to the degradatives of sulfur containing materials by enzyme. The sulfur containing materials in cruciferous plants, is catalyzed by plant and bacterial myrosinase [22]. So, needs to study for the role of lactic acid bacteria to the change of physiological activities in DLMJ are existed. Accordingly, this study was performed to know whether there is any change of physiological activity in DLMJ which is inoculated by lactic acid bacteria. Thus, physiological activities were examined for cytotoxicity, antioxidative, and ACE inhibiting activities.

MATERIALS AND METHODS

Preparation of DLMJ

The leaf mustard was obtained from Dolsan, Jeollanamdo, Korea, cut into 2 to 3 cm sizes, washed twice with distilled water and then crushed (Hanil, HMF-340, Seoul, Korea) and filtered through sterilized gauze. The filtrate was centrifuged at 1,500 rpm for 15 min, the supernatant filtered through a Whatman No. 2 paper. To reduced the compound of high molecules, DLMJ was sterilized by autoclave, further centrifuged at 3,000 rpm for 10 min and then used as the sample.

Microbes

The various lactic acid bacteria were isolated from 6-day-fermented DLMK at 20°C, which were then added to selective media. The *Leuconostoc* and *Weissella* groups were cultured in PES [23] medium at 26°C and the *Lactobacillus* and *Pediococcus* in LBS and *m*-Enterococcus agar broth at 37°C and 32°C, respectively. Single colonies from each medium were isolated, then restreaked on BUA (A microplate, Biolog, CA, USA) medium and identified using the Biolog system (MicroLog system, Biolog, CA, USA).

Reducing Sugar and Protein

The amount of reducing sugar and protein in S-DLMK by lactic acid bacteria were determined by the method of DNS [24] and Lowry [25], respectively.

16s rDNA Gene Sequence Analysis

A phylogenetic analysis was performed by 16S rDNA sequencing of *Weissella confusa* Dolsan selected from DLMK. The DNA was isolated by lysozyme and protease. Restriction endonuclase treatment of 3 μg DNA was performed, followed by electrophoresis. The forward and reverse primers were 5′-AGAGTTTGATCATGGCTCAG-3′, corresponding to position 27F, and 5′-GGATACCTT GTTACGACTT-3′, corresponding to position 1492R. Part of the rDNA operon, comprised of nearly complete 16S DNA, was amplified by PCR. The PCR-amplified 16S rDNA was purified using the QIAquick PCR purification kit and the sequence analysis using a DNA sequencer (Perkin-Elmer, ABI PRISM 3700, Swiss). The phylogenetic analysis of the 16S rDNA was performed using the GenBank and RDP database.

Inoculation of Lactic Acid Bacteria

The 7 isolated predominant strains were inoculated to the prepared DLMJ, and cultured for 48 h at 37°C, the cultured fluids centrifuged at 1,500 rpm for 15 min, and the pellet washed twice with saline solution. Cells (1×10^8 CFU/mL) were inoculated to the DLMJ and cultured at 37°C for 48 h. The culture was stopped until the time which was not changed the content of protein and sugar in DLMJ by lactic acid bacteria. Then, culture fluids centrifuged at 3,000 rpm for 15 min and the supernatants used as the samples.

Cell Culture

The cell line used was HepG2 (hepatocellular carcinoma, human, ATCC No. HB-8065), which was grown as a monolayer in RPMI-1640, supplemented with 10% FBS and 20 mM hepes buffer. The cells were maintained in a 5% CO_2 incubator (Forma Scientific Co., Model 3546, Marietta, USA) at 37°C .

MTT Assay

The adherent cells were dispersed as a single cell by treatment with trypsin-EDTA solution and dropped into a 96-well plate. Cells (1×10^4 cells/mL) in 200 μL of medium were added to each well and cultured for 24 h. After removing the medium, 20 μL of the samples were added to the 96-well plate and incubated at 37°C in a 5% CO_2 incubator for 72 h. After 72 h of culturing, 20 μL of freshly prepared MTT (5 mg/mL) solution [26] was added, and the plate incubated for a further 4 h at 37°C. 150 μL of isopropanol in 0.1 M HCl was added to the 96-well plate and left at room temperature for 30 min. The optical density was measured at 570 nm using a microplate reader (Biorad, Co., Benchmark, Germany). The percent cytolysis was calculated as follows: cytotoxicity (%) = $((C\text{-}S)/C)\times100$, where S is the absorbance of the sample and C that of the blank.

Antioxidative Activity

The antioxidative activity in DLMJ due to the addition of various lactic acid bacteria was determined using the DPPH (a′-diphenyl-a′-picrylhydrazyl) method [27]. 5 mL of the DPPH was added to 1 mL of sample and left to stand for 30 min. The absorbance of the resulting so-

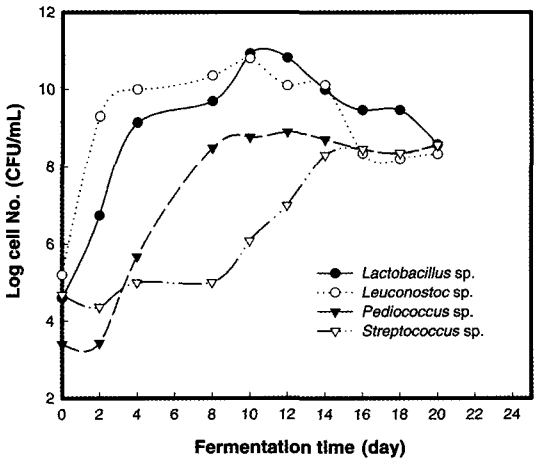

Fig. 1. Microflral changes of lactic acid bacteria in DLMK at 20°C.

lution was measured at 528 nm with a spectrophotometer (Shimadzu, UV-120A, Kyoto, Japan). Each sample was run four times, and the average calculated as the % antioxidative activity.

ACE Inhibiting Activity

Antihypertensive activities can be determined as the angiotensin I-converting enzyme (ACE) inhibiting activity. Angiotensin I-converting enzyme (ACE), a zinc containing enzyme, catalyzes the formation of the potent vasopressor angiotensin II from angiotensin I and inactivates bradykinin, which has a vasodilating action [28,29]. ACE a potent vasodilator, is also known as kininase II and is involved in the breakdown of kinins [30]. The ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) inhibiting activity was assayed by the method described by Cushman and Cheung, with slight modification [22]. The ACE and substrate for ACE, hippuryl-L-histidyl-L-leucine (Hip-His-Leu), were obtained from Sigma chemical Co.. The Hip-His-Leu was dissolved in 0.1 M of sodium borate buffer (pH 8.3). Then, 100 μL of 25 mM Hip-His-Leu solution was mixed with 50 μL DLMJ and preincubated for 10 min at 37°C. The reaction was initiated by the addition of 150 μL ACE dissolved in sodium borate buffer (pH 8.3), and the mixture incubated for 60 min at 37°C.

The reaction was stopped by the addition of 250 μL 1 M HCl. The hippuric acid was extracted with 1.5 mL ethyl acetate and the extracts centrifuged at 2,500 rpm for 10 min The supernatant was dried and dissolved in 3 mL 1 M NaCl The absorbance at 228 nm was measured to evaluate the degree of ACE activity inhibition. The ratio of the extent inhibition was calculated as follows: inhibitory ratio(%) = $((C\text{-}S)/(C\text{-}S'))\times100$, where S is the absorbance of the sample, S' that of control sample and C of the blank.

RESULTS AND DISCUSSION

Microfloral Changes

In a previous paper [21], the physiological activity in DLMK juice during the fermentation period increased significantly with an increase in the growth of microbes. In order to investigate the effect of the microbes on the physiological activity in DLMJ, the microbes in DLMK we isolated and identified using selective media. Fig. 1 shows the changes in the lactic acid bacteria in DLMK at 20°C. The pattern of the microfloral changes in each bacterial group, *Leuconostoc, Lactobacilli, Pediococci and Streptococci,* were similar to Chinese cabbage Kimchi [23]. During the optimum ripening period, the population of *Leuconostoc* and *Lactobacilli* were shown to be high, with maximum cell numbers, but later slowly decreased. However, the *Pediococci* and *Streptococci* had low populations throughout the fermentation period. The bacterial strains, isolated from the 10th day's DLMK fermented at 20°C, were identified using the Biolog system. Table 1 shows the metabolic activities of the isolated lactic acid bacteria in DLMK using 95 carbon sources.

Table 1. The metabolic activities of strains biochemically tested by the Biolog AN microplate assay

Carbon substrate	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Cellobiose	+*	+	+	+	+	+	+	+	B	+
Galactose	+	+	+	B	+	+	-	+	-	+
Glucose	+	+	+	+	+	+	+	+	B	+
Glycerol	-	+	b	+	-	-	-	-	B	-
Maltose	+	+	-	+	+	+	-	+	-	-
Mellibose	-	+	-	-	+	-	-	b	-	+
Lactose	b	b	-	-	+	+	-	b	-	b
Raffinose	-	+	-	-	b	-	-	-	+	-
L-rhamnose	b	-	-	-	-	b	-	b	-	-
Sucrose	+	+	+	+	+	+	-	b	+	+
Trehalose	-	+	+	+	+	-	+	+	B	+
Similarity index (SIM)	0.88	0.60	0.87	0.61	0.53	0.75	0.80	0.81	0.91	0.66

*Visually assembling color density, usually colorless : -, noticeable color : +, extremely faint color : b

D1 : *W. confusa*, D2 : *Leu. mesenteroides* sp. *mesenteroides*, D3 : *Lb. plantarum*, D4 : *Lb. alimentarius*, D5 : *Lb. raffinolactis*, D6 : *Lc. lactis*, D7 : *Lc. plantarum*, D8 : *P. pentosaseus*, D9 : *P. parvulus*, D10 : *Leu. gelidum*

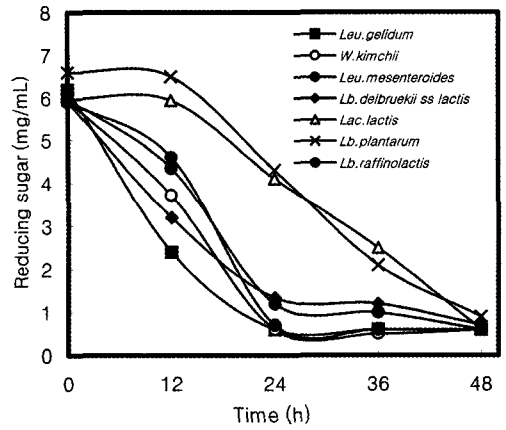

Fig. 2. Phylogenetic tree of isolated strain in DLMK.

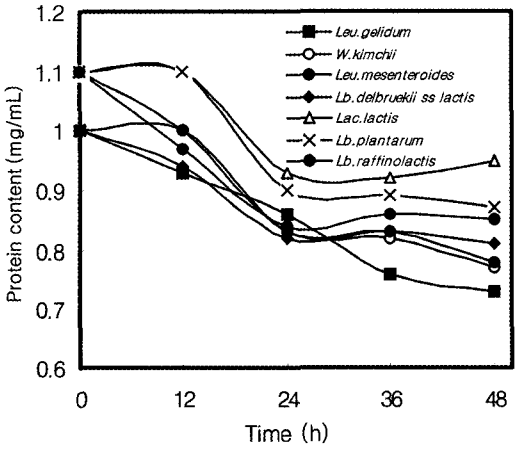

Fig. 3. Morphology of *W. kimchii* Dolsan.

The isolated strains of *Leuconostoc, Lactobacilli, Pediococci* and *Streptococci* were *Leuconostoc mesenteroides* sp. *mesenteroides, Leuconostoc gelidum, Weissella confusa, Lactobacillus plantarum, Lactobacillus alimentarius, Lactobacillus raffinolactis, Lactobacillus delbruekii* sp. *lactis, Lactococcus plantarum, Pediococcus pentosaceus* and *Pediococcus parvulus*, with the especially predominant strains called Dolsan.

16s rDNA Gene Sequence Analysis

Weissella confusa, as it is currently known, was previously known as *Lactobacillus confusus* and originally as *Lactobacillus coprophilus*. To clarify the identity of the phylogeny of *Weissella confusa* Dolsan, a phylogenetic analysis was performed using 16S rDNA sequencing.

Fig. 2 shows the phylogenetic tree of *Weissella confusa* Dolsan. The *Weissella kimchii* was the closest phylogenetic relative of *Weissella confusa* Dolsan, with a 16S rRNA similarity of 99.0%. Nam *et al.* reported that *Weissella confusa* has anti-*Helicobacter pylori* functions [20] and it is inferred from those reports that *Weissella kimchii* Dolsan also has physiological activity. Fig. 3 shows

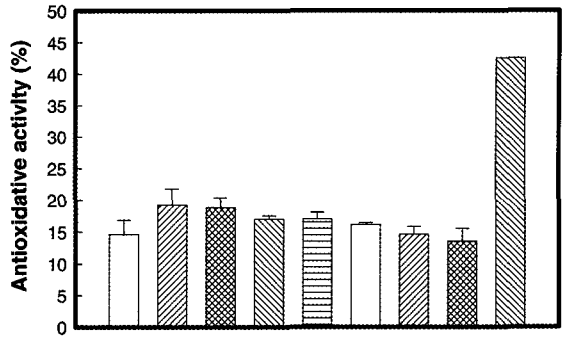

Fig. 4. Changes of reducing sugar in the juice of Leaf mustard by add of 7 lactic acid bacteria.

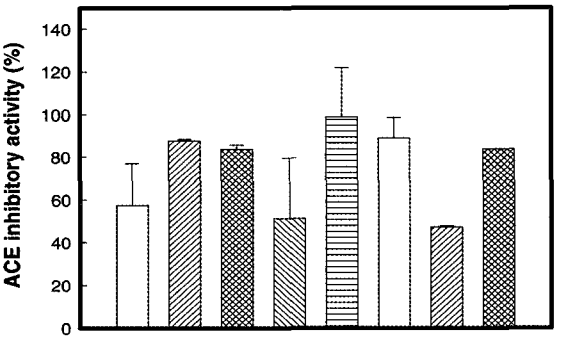

Fig. 5. Changes of protein content in the juice of Leaf mustard by add of 7 lactic acid bacteria.

the morphology of *Weissella kimchii* Dolsan. The shape of *Weissella kimchii* Dolsan was short-rod like, with a diameter shorter (0.45~0.8 μm) than *Weissella kimchii* (1~2 μm) [14].

Consequently, these results suggested that ACE activity inhibitory compounds are closely related to the metabolite production of extracellular enzymes by the *Weissella kimchii* Dolsan in DLMJ. However, the antihypertensive active compounds of *Weissella kimchii* Dolsan in leaf mustard remain unknown. Further studies will be needed to clarify the ACE inhibiting activity of DLMJ and identify and purify the active compounds in DLMJ.

Reducing Sugar and Protein

The changes of reducing sugar and protein contents in DLMJ by 7 isolated predominant strains were shown in Figs. 4 and 5, respectively. The hydrolysis ratio of reducing sugar and protein were higher in *Leuconostoc* species, like a *Leuconostoc mesenteroides* sp. *Mesenteroides* Dolsan, *Leuconostoc gelidum* Dolsan, and *Weissella kimchii* Dolsan than other species during 48 h. These results suggested that the possibility of *Leuconostoc* species having higher extracellar enzyme activity than other species. Choi *et al.* [20] has reported the isolation of various extracellular enzymes, such as α-amylase, β- amylase, protease, pectinesterase and polygalacturonase, produced by lactic acid bacteria from Kimchi. These re- sults suggested that

Table 2. The cytotoxicity of DLMJ on the addition of lactic acid bacteria

Sample	Cytotoxicity (%)
A	10.95 ± 4.21*b
B	18.15 ± 2.62a1)
C	10.98 ± 4.21b
D	2.35 ± 0.25d
E	18.60 ± 4.21a
F	19.55 ± 5.21a
G	11.70 ± 0.35b

*Mean ± S.D. (n=3)
1) Means with different letters beside the data are significantly, different at the 0.01 level of significance, as determined by Duncan's multiple range test
A: Dolsan leaf mustard juice (DLMJ)
B: DLMJ + *Leu. gelidum* Dolsan
C: DLMJ + *Leu. mesenteroides* sp. *mesenteroides* Dolsan
D: DLMJ + *Lc. lactis* Dolsan
E: DLMJ + *Lb. plantarum* Dolsan
F: DLMJ + *Lb. raffinolactis* Dolsan
G: DLMJ + *W. kimchii* Dolsan

the possibility of physiological hydrolysis compounds were produced in DLMJ by lactic acid bacteria. The degradation of glucosinolates, sulfur containing compounds in cruciferous plants, is catalyzed by plant and bacterial enzymes. And, these hydrolysis products of glucosinolates are known to have mutagenic, antimicrobial agent [5], and anticarcinogenic properties [31]. Accordingly, these results supported that the possibility of the production of naturally occurring compounds which are known to physiological compounds by lactic acid bacteria in DLMJ.

Cytotoxicity against HepG2

Table 1 shows the cytotoxicity toward the HepG2 of DLMJ by the addition of 7 isolated predominant strains. The cytotoxicities were higher than those of the control (DLMJ, itself), with the exception of *Lactococcus lacis* Dolsan inoculation. However, the cytotoxicities toward DLMJ by the addition of *Leuconostoc mesenteroides* Dolsan, *Lactobacillus delbrueckii lactis* Dolsan and *Weissella kimchii* Dolsan were all under 20.0%. DLM contains thiosulfates and organosulfur compounds, which are known to inhibit chemically-induced tumors [31]. In our previous studies, the water extracts of well-fermented leaf mustard Kimchi significantly inhibited the growth of cancer cells *in vitro* [32]. Also, on the addition of different parts of DLMJ at a concentration of 6% [33], the cytotoxicities against HepG2 were all over 50.0%. In this research, the cytotoxicity toward DLMJ by the addition of various lactic acid bacteria was not significantly high. This might have been due to the reduced cytotoxic active compounds, such as high molecular weight proteins adducted with sulfur containing materials during the sterilization of DLMJ.

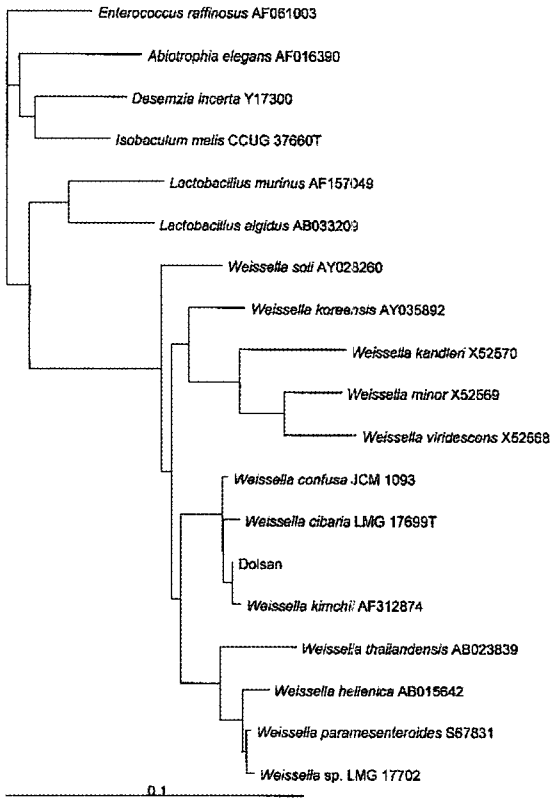

Fig. 6. Effect of lactic acid bacteria on antioxidative activity. A: *Lb. delbruekii lactis* Dolsan, B: *Leu. gelidum* Dolsan, C: *Lc. lactis* Dolsan, D: *Lb. plantarum* Dolsan, E: *W. kimchii* Dolsan, F: *Leu. mesenteroides* Dolsan, G: *Lb. raffinolactis* Dolsan, H: DLMJ, I: BHA (0.01%).

Antioxidative Activity

Fig. 6 shows the antioxidative activities due to the addition of 7 isolated predominant strains. The antioxidative activity toward DLMJ by the addition of *Leu. gelidum* Dolsan was higher than that of the other species. However, the antioxidative activities due to the addition of the various lactic acid bacteria were all under 20.0%. Leaf mustard contains high levels of antioxidative compounds, such as ascorbic acid, β-carotene and chlorophylls [1,2]. In this case, most of antioxidative activity toward DLMJ by the addition of various lactic acid bacteria was very low, which may be accounted for by the conversion of chlorophylls to pheophytins [34] and the degradation of carotenoides in DLMJ during the sample preparations.

ACE Inhibiting Activity

Fig. 7 shows the ACE inhibiting activity toward DLMJ by the addition of 7 isolated predominant strains. The ACE inhibiting activity toward DLMJ was 82.0%. As 7 isolated predominant strains were added to DLMJ, the ACE inhibiting activity increased more than that toward DLMJ. The ACE inhibiting activity toward DLMJ by the

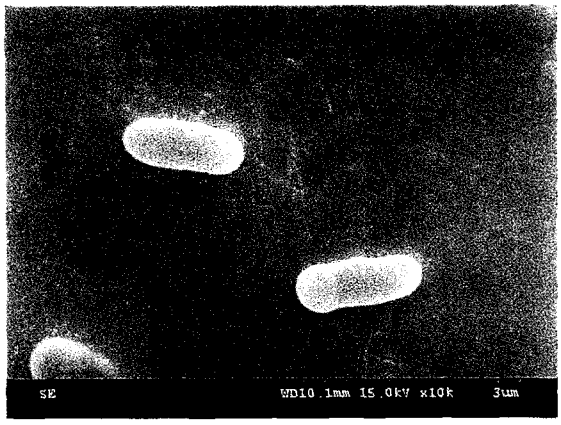

Fig. 7. Effect of lactic acid bacteria on ACE inhibitory activity. A: *Lb. delbruekii lactis* Dolsan, B: *Leu. gelidum* Dolsan, C: *Lc. lactis* Dolsan, D: *Lb. plantarum* Dolsan, E: *W. kimchii* Dolsan, F: *Leu. mesenteroides* Dolsan, G: *Lb. raffinolactis* Dolsan, H: DLMJ.

addition of *Leuconostoc mesenteroides* Dolsan, *Leuconostoc gelidum* Dolsan and *Weissella kimchii* Dolsan were 88.8, 87.7 and 94.0%, respectively. Hence, the DLMJ on the addition of *Weissella kimchii* Dolsan showed the maximum value of 94.0%.

The renin angiotensin system plays an important role in hypertensive diseases, and blocking this system with angiotensin converting enzyme (ACE) inhibitors, with natural sources, reduces hypertensive diseases in patients, which acts as protection and therapy. Recently, ACE inhibitors were screened from several natural sources, such as dried bonoto, sardine muscle, tuna, soy sauce and soybean paste (Doenjang). Yuk *et al.* [35] reported that ACE inhibitory effect of the pronase treated sample from *Sinapis alba* L. extracts decreased compared with that of the untreated sample. In our research [19], the digetion of protein by enzyme treatment was affected to ACE inhibiting activity, and ACE inhibiting was significantly related to the degradatives of sulfur containg materials. Cushman *et al.* reported [36] that two or tripeptide (Trp-Ala-Pro) *C*-terminals were very important as ACE inhibitors. These results suggested that sulfur containing materials in DLMJ was adducted with protein. These protein hydrolysis by lactic acid bacteria resulted to the reduction of sulfate in DLMJ. Consequently, the degradation of sulfur containing materials and the production of specific peptides in DLMJ by 7 isolated predominant strains gave rise to ACE inhibitory activity. Further studies are needed to analysis the content of sulfur containg materials and specific peptides in DLMJ by 7 isolated predominant strains.

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