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Changes of Physiological Activity of Mustard Leaf during Its Fermentation Period

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Abstract *In vitro* cytotoxicity and antioxidative effects of water extracts prepared from Mustard Leaf Kimchi (MLK) during its fermentation period were investigated. The cytotoxicity against HepG2 (human hepatic cancer) by water extracts from the well fermented Mustard Leaf Kimchi was higher than others (fermented for 0 to 6 days at 18°C), and IC_{50} of water extracts at the points of 6, 8, 10, and 14 days during fermentation were 213.4, 99.2, 99.9, and 109.8 μg/ml, respectively. Antioxidative activity of water extracts from MLK during various fermentation periods was higher than that of blank distilled water. However, the antioxidative activity of well-fermented water extracts of MLK (fermented for 8–14 days) did not show any difference from that of others (fermented for 2–6 days). Thus, water extracts of well-fermented MLK (fermented during 8–14 days) significantly inhibited the growth of cancer cells *in vitro*, but little antioxidative activity was influenced by the various fermentation periods.

Key words: Mustard Leaf Kimchi, cytotoxicity, antioxidative activity

Kimchi is a Korean traditional food and its ingredients have been investigated to determine it chemopreventive properties. Kimchi is also very popular not only for its taste but also for improving digestion, preventing constipation, controlling intestinal microflora, and for having pharmaceutical functions [17]. The raw materials of Kimchi consist of mainly green-yellow vegetables that are known to exhibit antimutagenic and anticancer activities [15]. Along with the fact that these have dietary fibers and lactic acid bacteria, kimchi can therefore also play a positive role in preventing colon cancer [22]. Lactic acid bacteria in Kimchi comprising the genera of *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and newly recognized *Carnobacterium*, contribute to the flavor, color development, and preservation of foods [13]. Lactic acid bacteria in Kimchi also show an antimicrobial activity by production of organic acids, hydrogen peroxide, carbon dioxide, and bacteriocins [2, 7, 9, 10, 12]. Mustard leafi, dentified as the major ingredient for Gat-Kimchi, is known for its unique flavor and hot taste. Since MLK is known to have antimicrobial effects on the microorganisms during fermentation, therefore, Mustard Leaf Kimchi (MLK) has a longer period of time to reach the optimum ripening state than that of other types of Kimchi [20]. Mustard leaf contains large amounts of thiosulfates and organosulfur compounds which are known to inhibit chemically-induced tumors [4]. Mustard leaf also contains high levels of ascorbic acid, β-carotine, chlorophylls, and other minerals such as calcium and potassium, etc. [5]. Ascorbic acid, β-carotine, and chlorophylls have some antioxidative activity [4]. Therefore, MLK is a potentially excellent food, since it exhibits anticancer and antioxidative effects. However, the anticancer and antioxidative activities of MLK during fermentation have been poorly elucidated until now. In this study, the water extracts of MLK during its fermentation period were investigated to determine the change of anticancer and antioxidative activities.

Materials and Methods

Preparation of Mustard Leaf Kimchi

To prepare Mustard Leaf Kimchi (MLK), mustard leaves grown in Dolsan, Jun-Nam, Korea were obtained. Garlic, ginger, red pepper powder, and green onion were purchased from a local market in Yosu, Korea. The mustard leaf was cut by 2 to 3 cm, brined in a 10% salt solution for 2 h, rinsed twice with distilled water, and then drained. The formulated ratio of ingredients [21] for MLK was 0.015 of garlic, 0.0005 of ginger, 0.015 of red pepper powder, and 0.015 of green onion of salted mustard leaf. The final salt concentration in the MLK was adjusted to 2%. The prepared MLK samples were put into vessels and

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fermented for 14 days at 18°C. This temperature was arbitrary selected.

Water Extracts of Mustard Leaf Kimchi (MLK)

MLK during various fermentation periods were collected, freeze-dried, and minced with a blender. The minced MLK was extracted three times with a 30-fold volume of boiling water (100°C) [16]. After extraction, the extract was filtered through Whatman No. 2 paper and dried by a freeze dryer.

Cell Culture

The cell line used was HepG2 (Hepatocellular carcinoma, human, ATCC). The cells were grown as a monolayer in RPMI-1640 (Sigma Co., U.S.A.) medium supplemented with 10% FBS and 20 mM Hepes buffer, and were maintained in a 5% CO_2 incubator (Model 3546, Forma Scientific, U.S.A.) at 37°C.

Cytotoxicity Assay

The adherent cells were dispersed into a single cell by treatment with trypsin-EDTA solution and were then dropped into a 96-well plate. Cells (1×10^4 cells) in a 200 µl medium were added into each well and cultured for 24 h. After removing the medium, various concentrations (0, 63, 125, 250, 500, 1000 µg/ml) of water extracts were added into the wells, and they were incubated at 37°C in 5% CO_2 incubator for 72 h. After the culture, 20 µl of freshly prepared MTT (5 mg/ml) solution [19] was added and the plate was incubated for another 4 h at 37°C. One-hundred-and-fifty µl of 0.1 N isopropanol in HCl was added into a 96-well plate and the microplate was left at room temperature for 30 min. The optical density was measured at 570 nm by using the microplate reader (Benchmark, BioRad Co., Germany). The percent of cytotoxicity was calculated as follows:

$$\frac{Ab_{570}\ (\text{without sample}) - Ab_{570}\ (\text{with sample})}{Ab_{570}\ (\text{without sample})} \times 100$$

Antioxidative Activity

The antioxidative activity of water extracts of MLK during various fermentation periods was determined by the α,α'-diphenyl-β-picrylhydrazyl(DPPH) method [14]. Five ml of DPPH was added to 1 ml of 0.1% water extracts and the activity was determined by decrease of absorbancy at 528 nm.

Determination of pH, Acidity, and Total Number of Microbes

The blended MLK sample (20 g) was added to 180 ml of distilled water and filtered on Whatman No. 2 paper. The pH of the filtrate was determined with a pH meter (Model 735P, Istek, Korea) and acidity was determined according to the method of AOAC [1]. One-tenth % of phenolphtalein in ethanol solution was dropped into the filtrate and titrated with 0.1 N NaOH. Then, the lactic acid content was calculated and expressed as the % acidity.

The total number of microbes was determined by the pouring method: The broth of MLK was diluted from $1/10^6$ to $1/10^8$, and the diluted sample was inoculated into TGY (Trypton glucose yeast extracts) solid medium [18]. The plates were incubated at 30°C for 48 h, and the total cell number was estimated as a colony forming unit (cfu/ml).

Determination of Reducing Sugar and Protein in Water Extracts of MLK

The amount of reducing sugar and protein in water extracts of MLK were determined by the method of DNS [8] and Lowry [3], respectively.

Results and Discussion

Changes of pH, Acidity, and Total Number of Microbes

Changes of pH and acidity in MLK during fermentation at 18°C are as shown in Fig. 1. The initial pH of MLK was 5.74, decreasing to 4.12 after 14 days of fermentation at 18°C. The initial acidity expressed as lactic acid content in MLK was 0.18%, and it increased to 0.85% after 14 days. In the case of Chinese cabbage Kimchi [6], the optimum ripening acidity (0.8%) and pH (4.0) were reached within 4 days at 17±1°C, however, MLK reached the optimum ripening acidity (0.85%) and pH (4.12) within 14 days at 18°C. Thus, the time of MLK reaching the optimum ripening state appeared to be longer. Figure 2 presents the changes seen in the total cell numbers during 14 days of fermentation at 18°C. The time taken to reach the maximum cell density in MLK was 6 days and then the cell number slowly decreased. In terms of Chinese

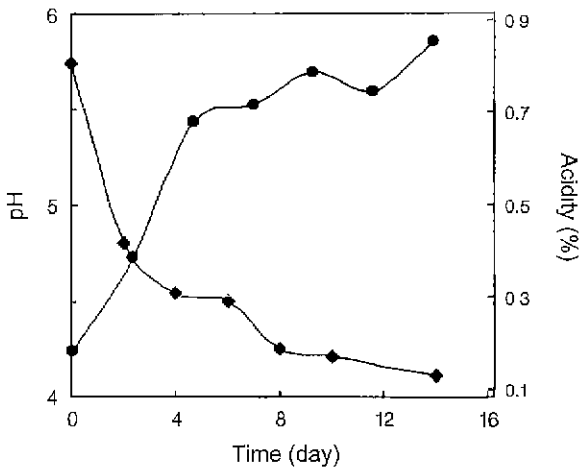

Fig. 1. Changes of pH and acidity (%) in MLK during fermentation at 18°C.
◆: pH; ●: Acidity (%)

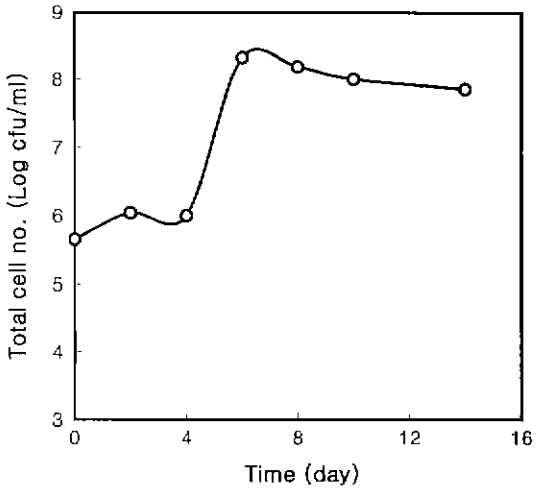

Fig. 2. Changes of the total cell number of microbes in water extracts from MLK during fermentation at 18°C.

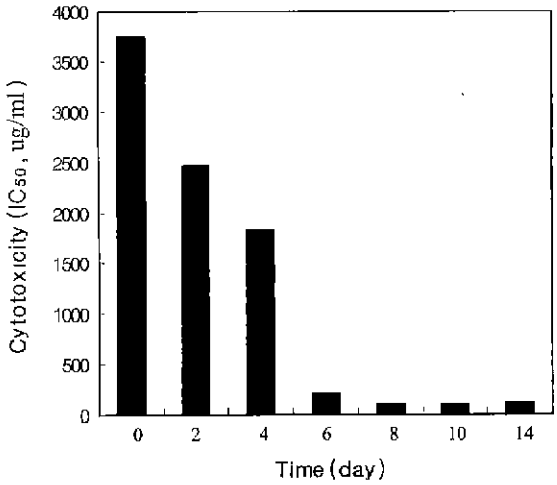

Fig. 3. Cytotoxic effect of water extracts according to various fermentation periods on HepG2.

cabbage Kimchi [6], the time taken to reach the maximum cell density was 2 days at 17±1°C and, thereafter, the cell numbers were reduced more rapidly than that in MLK. During the optimum ripening period, the number of total cells rose to its highest level with good taste [11]. The above data showed that MLK was much slower in reducing pH and increasing the acidity as compared to those of Chinese cabbage Kimchi. Therefore, the fermentation period required was 3-5 times of that of Chinese cabbage Kimchi [20].

Cytotoxic Effects

The cytotoxic effect of MLK water extracts during various fermentation periods of HepG2 is shown in Table 1. The cytotoxicity by the water extracts of well-fermented MLK (8-14 days) against HepG2 was higher than others (0-6 days). Park [17] reported that Chinese cabbage Kimchi had an anticancer activity, and that the properly ripened Kimchi had a higher potency of preventing cancer compared to fresh Kimchi. The IC_{50} of water extracts of MLK fermented for 0, 4, 10, and 14 days were 3329, 1840, 99.9, and 109.8 μg/ml, respectively (Fig. 3). Conclusively, well-fermented MLK seems to have stronger cytotoxicity than fresh MLK. These results might have resulted from active compounds produced during the fermentation process.

Table 1. Comparison of cytotoxic effects of water extracts* of MLK on the growth of HepG2 using the MTT assay.

Fermentation period (d)	Cytotoxic effect (%)
0	20.6
2	31.6
4	26
6	55
8	62.4
10	66.7
14	59.4

*The concentration of water extracts of MLK was 500 μg/ml.

Antioxidative Activity of Water Extracts of MLK

Water and methanol extracts of Chinese cabbage Kimchi had a considerable amount of antioxidative activity to inhibit peroxides formation [16]. Hence, we also examined the antioxidative activity of MLK during various fermentation periods. The antioxidative activity of water extracts of MLK during the fermentation process is shown in Fig. 4. Our results showed that water extracts (0.1%) had a strong antioxidative activity comparable to that of the blank (distilled water), but the activity was weaker than that of BHA (0.01%), a reference compound. However, the

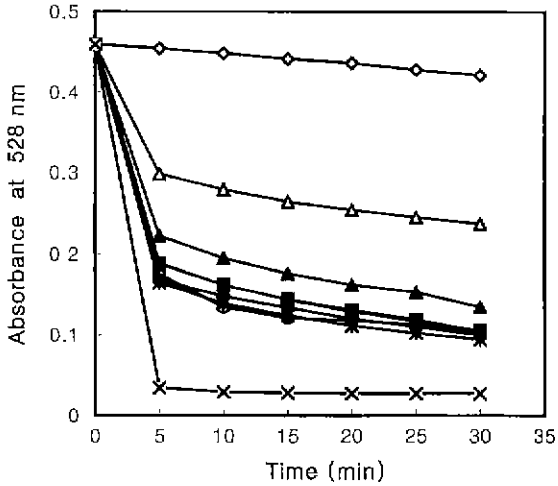

Fig. 4. Changes of the free radical level of DPPH by the addition of water extracts according to various fermentation period.
◇: blank; □: 0 day; △: 2 day; ●: 4 day, ○: 6 day, ▲: 8 day, ■ 10 day, +: 14 day; ×: BHA

antioxidative activity of water extracts from well-fermented MLK (fermented for 8–14 d) did not show any difference from those of others (fermented for 2–6 d). Consequently, the fermentation period did not affect the antioxidative activity of MLK.

Changes of Protein and Reducing Sugar Contents in Water Extracts of MLK

Antioxidative activity was hardly affected by the fermentation period of MLK. On the contrary, well-fermented MLK significantly inhibited the growth of cancer cells *in vitro*. The changes of protein and reducing sugar contents in water extracts of MLK were further examined during the fermentation period, and the results are shown in Fig. 5. It took 6–10 days to reach the maximum level of protein content in water extracts of MLK, and it then slowly decreased for 14 days. Coincidently, this is in good agreement with the tendency shown in Fig. 2. The reducing sugar content in water extracts of MLK is shown in Fig. 6. The level of reducing sugar gradually decreased as the cell numbers increased during fermentation. Generally, fermentation of Kimchi is carried out by microorganisms, mainly the lactic acid bacteria. Lactic acid bacteria, which are abundant in well-fermented Kimchi, are known to have an anticancer activity, therefore, it can be stated that water extracts of well-fermented MLK might possibly contain high levels of proteins produced by lactic acid bacteria, causing high levels of cytotoxicity against cancer cells. Further studies are needed to clarify on the cytotoxic effect, and to identify and purify the active compounds in the water extracts of well-fermented MLK.

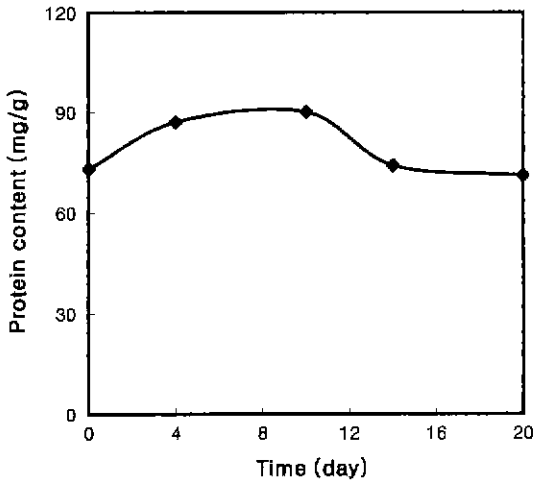

Fig. 5. Changes of protein content in water extracts from MLK during fermentation at 18°C.

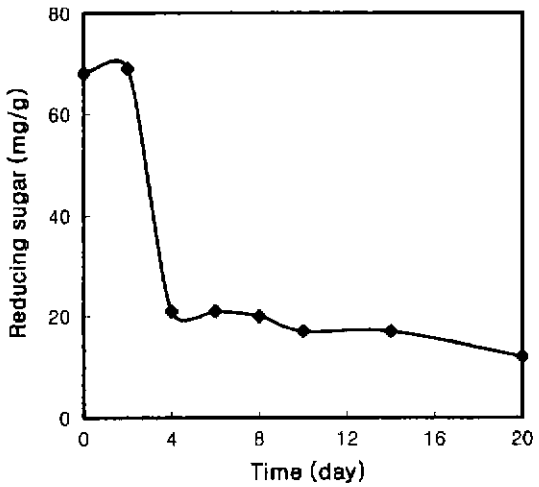

Fig. 6. Changes of reducing sugar in water extracts from MLK during fermentation at 18°C.

Acknowledgments

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References

1. AOAC. 1995. Fruits and products acidity (titrable) of fruit products. *Official Methods of Analysis*. Chapter 37, p. 10. 16th ed. AOAC International, U.S.A.
2. Cha, D. S. and D. M. Ha. 1996. Isolation of *Leuconostoc mesenteroides* subsp. *mesenteroides* DU-0608 with antibacterial activity from Kimchi and characterization of its bacteriocin. *J. Microbiol. Biotechnol.* **6:** 270–277.
3. Cheigh, S. K. 1997. *Food Analysis (Standard)*, pp. 297–298. Ji-Gu Publishing Co., Korea.
4. Cheigh, H. S. and K. Y. Park. 1994. Biochemical, microbiological and nutritional aspects of Kimchi (Korean fermented vegetable products). *Crit. Rev. Food Sci. Nutr.* **34:** 175–203.
5. Cho, Y. S. and S. K. Park. 1990 Studies on the chemical components of Korean *Brassica Juncea Cosson*. *Sunchon Natl. Univ. Bull.* **9:** 167–175.
6. Choi, S. Y., M. K. Lee, K. S. Choi, Y. J. Koo, and W. S. Park. 1998. Changes of fermentation characteristics and sensory evaluation of Kimchi on different storage temperature. *Kor. J. Food Sci. Technol.* **30:** 644–649.
7. Choi, Y. O. and C. Ahn. 1997. Plasmid-associated bacteriocin production by *Leuconostoc* sp. LAB145-3A isolated from Kimchi. *J. Microbiol. Biotechnol.* **7:** 409–416.
8. Gail, L. M. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426.
9. Ha. D. M., D. S. Cha, and S. G. Han. 1994. Identification of bacteriocin-producing lactic acid bacteria from Kimchi and partial characterization of their bacteriocin. *J. Microbiol. Biotechnol.* **4:** 305–315.
10. Kim. S. K., E. J. Lee, K. Y. Park, and H. K. Jun. 1998. Bacteriocin produced by *Lactobacillus curvatus* SE1 isolated from Kimchi *J. Microbiol. Biotechnol.* **8:** 588–594.

11. Kim, Y. C., E. Y. Jung, H. J. Jung, S. G. Hong, K. T. Jung, and S. M. Kang. 1999. Improvement of Kimchi fermentation by using acid-tolerant mutant of *Leuconostoc mesenteroides* and aromatic yeast *Saccharomyces* fermentation as starters. *J. Microbiol. Biotechnol.* **9:** 22–31.
12. Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* **70:** 337–349.
13. Lee, H J., C. S. Park, Y. J. Joo, S. H. Kim, J. H. Yoon, Y. H. Park, I. K. Hwang, J. S. Ahn, and T. I. Min. 1999. Identification and characterization of bacteriocin producing Lactic acid bacteria isolated from Kimchi. *J. Microbiol. Biotechnol.* **9:** 282–291.
14. Lee, J. S and H. S. Cheigh. 1997. Antioxidative characteristics of isolated crude phenolics from soybean fermented foods (Doenjang). *J. Kor. Soc. Food Nutr.* **26:** 376–382.
15. Lee, K. I. 1992. Inhibitory effects of green-yellow vegetables of mutagenicity induced by various mutagens and on the growth of human cancer cells. Ph.D. Thesis. Pusan National University. Pusan, Korea.
16. Lee, Y. O. 1996. Studies on the antioxidative characteristics and antioxidative substances of Kimchu. Ph.D. Thesis. Pusan National University. Pusan. Korea.
17. Park, K. Y. 1995. The nutritional evaluation, and antimutagenic and anticancer effects of Kimchi. *J. Kor. Soc. Food Nutr.* **24:** 169–182.
18. Peterson, C. S. and M. H. Albery. 1969. The sauerkrant fermentation. New York state, Agr. Exp. Station. Geneva, Cornell Univ. Bull. No. 824.
19. Scudiero. D. A., R. H. Shoemaker, K. D. Paull. A. Monks, S Tierney, T. H. Nofiziger. M. J. ns, Currre. D. Seniff. and M. R. Boyd. 1988 Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **48:** 4827–4832.
20. Song. E. S., Y. S. Jeon, and H. S. Cheigh. 1997. Changes in chlorophylls and carotenoides of Mustard leaf Kimchi during fermentation and their antioxidative activities on the lipid oxidation. *J. Kor. Soc. Food Nutr.* **26:** 563–568.
21. Son, Y. M., K. O. Kim, D. W. Jeon, and K. H. Kyung. 1996. The effect of low molecular weight chitosan with and without other preservatives on the characteristics of Kimchi during fermentation. *Kor. J. Food Sci. Technol.* **28:** 888–896.
22. Wargovich, M. J., C. Wood. V. W. S Eng, L. C. Stephens, and K. Gray, 1988. Chemoprevention of N-nitrosomethylbenzylamine induced esophageal cancer in rats by the naturally occurring thioether, diallyl sulfide. *Cancer Res.* **48:** 6872–6875.