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Tumor Cell Proliferation Inhibitory and Antioxidative Activities of Spore Forming Lactic Acid Bacteria

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포자생성 유산균의 종양세포 증식 억제 활성과 지방산 산화 억제 활성

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요 약

포자형성 유산균주에 대하여 종양세포에 대한 증식 억제 활성과 항산화 활성 및 세포내 glutathione 함유율을 측정하였다. 종양세포에 대한 억제활성은 균주에 따라 큰 차이를 나타내었고 *Bacillus coagulans* KTCC 3675는 높은 수준의 특이한 수준의 억제 활성을 나타내었고 human lymphoma cell NCLH-1299에 대한 억제 활성은 미약한 수준인 것으로 확인되었다. TBA 방법에 의한 linoleic acid에 대한 항산화 활성은 5~25% 정도의 항산화 활성을 보였고 특별히 높은 수준의 항산화 활성을 보인 균주는 *Bacillus coagulans* KTCC 625, *Bacillus coagulans* KTCC 1015 및 *Lactobacillus sporogens* CU 815 인 것으로 확인되었다. 포자형성 유산균체 추출액의 linoleic acid 과산화 억제 활성과 세포내 glutathione 함유율과의 상관계수는 0.78로서 고도의 유의성이 인정되는 상관계수를 나타내었다.

(Key words : Tumor cell proliferation inhibition, GSH level, Peroxidation inhibition)

I. Introduction

Probiotics areused as viable microbial feed supplements that affect the host animal improving its intestinal microbial balance (Fuller, 1992). The addition of the feed of probiotics or antibiotics, that are competitive with pathogenic bacteria to the digestive tract, improves the performance of the animals, especially under stress conditions eg, weaning and dietary changes for piglets. Beneficial effects on the domestic animals include improved general health, more efficient feed utilization, faster growth rate, and increased milk and meat production.

Lactobacilli and bifidobacteia are considered to have several beneficial physiological effects, such as antimicrobial activity, enhancing of immune potency and antitumorogenic activities (Fuller, 1992; Salminen et al., 1998). It has been shown that some lactobacilli possess antioxidative activity, and are able to decrease the risk of accumulation of reactive oxygen species (ROS) during the ingestion of food (Kaizu et al., 1993). The tripeptide GSH is the major non protein thiol compound in biological systems; GSH is found in all eukaryotic cells,

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where it is involved in a variety of cellular functions (Kosower and Kosower, 1978; Meister and Anderson, 1983; Anderson, 1985) but relatively little is known in procryotes. The biological function of GSH in bacteria are not completely understood, but GSH may serve a protective role when toxic compounds are present (Apontoweil and Berend, 1975; Romero and Canada, 1991).

Several strains of *Bacillus* have shown promising characteristics (Cavazzoniand Adami, 1993; Kumprecht and Zobac, 1996). However there has been no thorough investigation into performance due to probiotic(Fuller, 1992), few studies have been carried out on spore forming lactic acid bacteria as probiotic, they are a group of Grampositive bacteria, sharing characteristics common to the genera *Bacillus* (spore forming, motile) and *Lactobacillus* (Suzuki and Yamamoto, 1994; Yoon and Won, 2002). This group includes *B. coagulans*, (Hammer, 1915), *B. lacemilacticus*, and *B. laevolacticus* and members of the genus *Sporolactobacillus* (Doores and Westhoff, 1983; Holzafel and Botha, 1988). They have many advantages over *Lactobacillus* spp. eg they possess the capacity to sporulate and in the spore form they are more resistant to heat, which facilitates the pelleting process used in the mass production of probiotic animal feeds. Little is known on thetumor cell proliferation inhibitory activity, antioxidative activity and glutathione content of spore forming lactic acid bacteria.

The objectives of this study were to investigate the inhibitory effects on the proliferation of carcinoma cell lines, the inhibitory activity in the peroxidation in the linoleic acid oxidation system and the level of cellular GSH in their cytoplasm of spore forming lactic acid bacteria.

Ⅱ. Materals and Methods

1. Bacterial Strains, Culture Conditions and Carcinoma Cell Lines

Bacillus coagulans CU 819 was isolated in our laboratory from cattle feces, other strains used in this investigation included *Bacillus coagulans* KCTC 1015, KCTC 1805, KCTC 1807, KCTC 1809 , KCTC 1823, KCTC 3825, KFRI 841 *Sporolactobacillus inulinus* ATCC13538, *Lactobacillus sporogenes* CU815 and *Bacillus polyfermenticus* SCD BP-1. 9 strains were isolated from the mud samples and identified by the API kit as *Bacillus megaterium* KM1, *Bacillus circulans* KM2, *Bacillus stearothermophilus* KM3, *Bacillus circulans* KM4, *Bacillus licheniformis* KM5, *Bacillus megaterium* KM6, *Bacillus circulans* KM9, *Bacillus pumilus* KM10 and *Bacillus licheniformis* KM17. Strains were grown at 37℃ in *Bacillus coagulans* medium (Difco Laboratories, Detroit, MI, USA). *Bacillus coagulans* and *Sporolactobacillus inulinus* were incubated under anaerobic conditions in water jacketed incubator. Cultures were maintained in MRS and *Bacillus coagulans* broth with 20% sterile glycerol and stored at −80℃.

Cell lines of WiDr(human colon carcinoma), Caski (human cervix carcinoma), CMT-93(mouse rectum carcinoma), A-498(human kidney carcinoma), MKN-45 (human stomach carcinoma), NCL-H1299(human lymph node) SNU-C1(human colon carcinoma) were used in this study. The cell lines were maintained in RPMI-1640 (GIBCO BRL, Grand Island, NY, USA) medium with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA) in a humidified incubator with 5% CO_2 at 37℃.

2. Colorimetric MTT Assay for Carcinoma Cell Proliferation Inhibition by Spore Forming Lactic Acid Bacteria

Bacterial cells were cultured in appropriate medium at optimal cultivation temperature as indicated in the bacterial strains for 24 hours, the culture were centrifuged at 10,000 ×g for 10min and washed twice with deionized water. Cells were resuspended in deionized water, ultrasonic disruption sonication was performed for three 1-min intervals in an ice bath. Cell debris was removed by centrifugation at 8,000 ×g for 10 min, and the resulting supernatant was the intracellular cell free extract(Mosman, 1983).

100㎕ of suspension (1106 cells /ml) and 60㎕ of medium was added to appropriate wells of sterile 96 well flat bottomed microtitre plates, and incubated for 24 h for cell stabilization. 40㎕ of spore forming lactic acid

bacterial cell free extract were added and incubated for 72h. At 68h of incubation, MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma M2128) was dissolved in PBS at 5mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. 10 ㎕ of MTT solution were added to each well, and incubated for another 6~8 h. Cells from some samples would not adhere to plastic and when this occurred the plates were centrifuged at 200 × g for 10 min prior to the removal of mixture at each stage of the assay. Approximately 150㎕ of media was removed and then the formazan crystal was dissolved in 150㎕ of dimethyl sulfoxide (DMSO) by shaking 5 min and incubated for 60min. The plates were then read on a microplate reader using a test wave length of 570 nm. The survival rate of tumor cells to each cell lysates was calculated as following;

Survival rate(%);
〔1 - (OD of sample treated cells/OD of control cells)〕 × 100

3. Determination of Inhibitory Activities of Linoleic Acid Peroxidation

Total antioxidative activity (TAA) of the test bacteria was assessed by using the linoleic acid test. The standard of linoleic acid in 96% ethanol (1:100) was diluted in isotonic saline (1;125). To the 0.4 ml linoleic acid, diluted in isotonic saline, was added 0.01% sodium dodecyl sulfate(lauryl sulfate L 5750, Sigma) and the sample (0.045ml of lactobacilli cell lysates or cells). The incubation was started by adding 0.1 mM $FeSO_4$(F 7002, Sigma) and the mixture was incubated at 37℃ for 60min. Then the reaction was interrupted by adding 0.25% butylated hydroxyltoluene (B-1378, Sigma) and the mixture was treated with 0.5ml acetate buffer (pH 3.5) consisting of acetic acid glacial and sodium acetate trihydrate(A-6283 and S-8625 respectively, Sigma) and heated with freshly prepared 1% thiobarbiturate solution (TBA)(T-5500, Sigma) at 80℃ for 40 min. After cooling, the mixture was acidified by adding 0.5 ml cold 5M HCl, extracted with 1.7 ml cold 1-butanol(BT 105, Sigma) and centrifuged at 3,000 ×g for 10 min and absorbance of butanol fraction was measured. The TAA of sample was expressed as the inhibition by sample of LA standard peroxidation as follows; (1 - (A 534(sample)/ A 534(LA as control) × 100. The higher numerical value (%) of TAA indicates the higher TAA of the sample. Peroxidation of LA-standard in the isotonic saline (without sample) served as a control.

4. Measurement of Glutathione Sulphydryl Content in Intracellular Cell Free Extract

Measurement of glutathione sulphydrylcontent in intracellular cell free extract of probiotic strains was performed using the GSH assay kit (calbiochem, Cat. No.354102. USA) took 200㎕ of intracellular cell free extract and adjusted the total volume to 900㎕ with buffer (solution 3) and added 50㎕ of solution R 1 and mixed thoroughly, and added 50㎕ of solution R2 andmixed thoroughly and then incubated the sample at 25 ℃ for 10 min in the dark and measured the final absorbance (A) at 400 nm and the calculation was performed using the following equation;

$$[GSH] = \frac{(A - Ao) \times D}{\varepsilon}$$

where [GSH] is the glutathione concentration in the sample expressed as molar concentration, A and Ao are absorbances measured in the presence and in the absence of sample respectively, ε is the apparent molar extiction coefficient of the product of the standards measured at 400 nm and D is the dilution factor of the sample.

5. Statistical Analysis

Within the same treatment group, for the comparison of the tumor cell proliferation inhibitory, activities, the percentage of inhibition of linoleic acid peroxidation, and glutathione content values between the treatments values were compared using SAS Duncan's multiple-range test. Spearmans' rank correlation quotient between the carcinoma cell proliferation inhibitory activity and the linoleic acid peroxidation inhibitory effects and that

between the tumor cell proliferation inhibition and the cellular GSH levels were calculated and the significance of the quotient was analyzed.

Ⅲ. Results and Discussion

1. Colorimetric Assay for Carcinoma Cell Growth Inhibition by Spore Forming Lactic Acid Bacteria

Results of colorimetric assay for carcinoma cell growth inhibition by spore forming lactic acid bacteria were shown in Table 1.

Bacillus coagulans KTCC 3625 has shown a marked antiproliferative effect against all the carcinoma cells and the isolated strain *Bacillus circulans* KM4 also has been selected as a strain which had made a strong inhibitory effect upon the carcinoma cells. But *Bacillus coagulans* KCTC 1823, *Bacillus coagulans* KFRI 841 and *Bacillus stearothermophilus* KM 3 have not made any inhibitory activity against the carcinoma cells tested. The least

Table 1. Percent of inhibition of cytoplasm fractions of *Bacillus coagulans* against various tumor cell lines (Inhibition%)

Cell line / Bacillus	WiDr	CMT-93	A-498	NCL-H1299	Caski	SNU-C1	MKN-45
B. coagulans KCTC 1807	37.5±1.3^{d}	-	10.8±1.0^{i}	-	19.5±1.0^{e}	22.7±0.6^{b}	-
B. coagulans KCTC 1809	31.8±1.0^{e}	15.9±0.3^{e}	19.4±0.7^{f}	18.7±0.3^{b}	27.2±0.9^{c}	-	20.9±1.4^{d}
B. coagulans KCTC 819	1.2±0.1^{k}	6.6±0.1^{f}	5 ±0.2^{l}	-	10.3±0.3^{h}	10.3±0.9^{f}	-
B. coagulans KCTC 1805	-	2.7±0.1^{i}	4.6±0.5^{l}	0.3±0.1^{g}	-	-	2.9±0.2^{h}
Lb. sporogenes CU815	40.2±0.9^{c}	4.8±0.4^{h}	19.3±0.4^{c}	17.4±0.6^{c}	34.2±0.3^{a}	-	-
B. coagulans KCTC 36	41.5±0.4^{b}	29.4±0.7^{a}	35 ±0.2^{b}	22.1±0.2^{a}	19.2±0.7^{e}	36.8±0.4^{a}	9.3±0.3^{f}
B. polypermenticus SCD BP-1	56.7±0.7^{a}	22.1±1.0^{b}	2.5±0.7^{m}	-	33.9±0.2^{a}	-	4.3±0.8^{g}
B. coagulans KCTC 1015	2.1±0.1^{k}	4.6±0.2^{h}	15.8±1.0^{g}	-	7.9±0.1^{i}	9.2±1.0^{g}	10 ±1.4^{f}
B. coagulans KCTC 1825	-	-	-	-	-	-	-
B. coagulans KFRI 841	-	-	-	-	15.5±0.7^{f}	-	-
Sl. inulinus ATCC13538	8.6±1.2^{j}	3.5±0.3^{i}	-	-	11.5±1^{gh}	5.3±1.1^{h}	0.1±0.1^{i}
B. megaterium KM1	15.1±0.3^{h}	5.6±0.5^{g}	7.9±0.1^{k}	8.3±0.1^{e}	26.8±5.0^{d}	15.1±0.7^{e}	18.7±0.8^{d}
B. circulans KM2	-	-	-	-	-	-	0.7±0.3^{i}
B. stearothermophilus KM3	-	-	-	-	-	-	-
B. circulans KM4	25.8±1.0^{f}	15.7±0.4^{e}	25.5±0.7^{d}	12.5±0.2^{d}	30.5±0.8^{b}	19.5±1.0^{c}	14.9±0.1^{e}
B. licheniformis KM5	-	-	39.6±1.0^{a}	-	-	-	31.1±1.0^{ab}
B. megaterium KM6	18.1±0.1^{g}	-	11.8±1.0^{h}	3.5±0.6^{f}	-	18.5±0.4^{c}	-
B. circulans KM9	18.5±0.4^{g}	17.8±1.2^{d}	25.5±0.9^{d}	-	14.8±0.7^{f}	18.5±1.0^{c}	30.6±0.7^{a}
B. pumilus KM10	13.4±0.2^{i}	15 ±1.0^{e}	23.2±0.9^{e}	-	13.4±0.2^{fg}	16.9±0.9^{d}	28 ±1.0^{c}
B. licheniformis KM17	14.3±0.4^{h}	20 ±1.0^{c}	26.9±0.1^{c}	-	14 ±0.1^{f}	18.9±0.8^{c}	29 ±1.0^{bc}

-: no antiproliferative effect at 40㎍/40㎕.

inhibition has been displayed by the spore forming lactic acid bacteria upon the NCL-H1299 human lymphoma cell line. The average inhibitory activity of spore forming lactic acid bacteria was weaker than those of *Lactobacillus* spp. Kim (2002) reported cellular components of lactic acid bacteria revealed antiproliferative effects on various human tumor cell lines, peptidoglycan and the cytoplasm fraction of lactic acid bacteria and the heat-killed whole cells also showed significant antiproliferative activities against several tumor celllines. In particular, cytoplasm fraction *in vitro* exhibited marked direct antiproliferative activities against cancer cell lines. The effect varied markedly depending upon the species and strains of spore forming lactic acid bacteria.

A tetrazolium salt has been used to develop quantitative colorimetric assay for mammalian cell survival and proliferation. Studies of cell biology in the 1960s (Slater et al, 1963) showed that the succinate dehydrogenase in the respiratory chain of mitochondria of living cells can oxidize succinic acid to fumaric acid through dehydrogenatin. As a hydrogen receptor, the tetrazolium salt is reduced to purple formazan. MTT is cleaved by all living metablolically active cells but not by dead cells or erythrocytes. The amount of formazan generated is proportional to the cell number. Activated cells produce more formazan than resting cells. The reduction of MTT to formazan product appears to be carried out by all the cell types such as myeloma, lymphoma and tumor cell lines. The main advantage of the colorimetric assay are the rapidity and precision, MTT assay may have very wide applicability for measuring survival and proliferation of cells.

2. Lipid Peroxidation Inhibitory Effects of Spore Forming Lactic Acid Bacteria

As shown in Fig. 1 and 2, all the *Bacillus* and other spore forming strains demonstrated an inhibitory effect on linoleic acid peroxidation. The inhibitory rates on linoleic acid peroxidation ranged from 5.0 to 65.0 % when intracellular cell free extract was added. The results indicated that all strains tested demonstrated antioxidative activity for inhibiting lipid peroxidation, and an extensively high degree of inhibitory activity on linoleic acid peroxidation occurred by three strains of *Bacillus coagulans* KTCC3625, *Bacillus coagulans* KTCC1015 and *Lactobacillus sporogens* CU 815. There exists some variation among the strains of *Bacillus coagulans* their inhibitory activity on linoleic acid peroxidation. Lipid peroxidation may results in toxic compounds, hydroperoxides are the primary initial prducts of lipid autoxidation, which is the major reaction involved in autoxidative deterioration of lipids. Hydroperoxides are are potentially toxic and capable of damaging DNA (Baker and He, 1991). Malonaldehyde is a secondary product of lipid peroxidation, it is highly reactive substance that can cause deterioration of biological molecules such as proteins and DNA(Aubourg, 1993). The capability forscavenging malondialdehyde by *L. acidophilus* and *B. longum* may provide an effective protection against this toxic product of lipid peroxidation.

Spearmans' rank correlation quotient between the carcinoma cell proliferation inhibitory activity and linoleic acid peroxidation inhibitory effects of the spore forming lactic acid bacteria was shown to be 0.05 which means

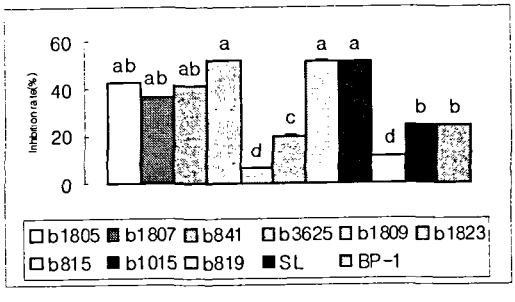

Fig. 1. Lipid peroxidation inhibitory activities of spore forming lactic acid bacteria of stock cultures.
Legend of strain *B. coagulans* CU 819 (b819), *B. coagulans* KCTC 1015 (b1015), *B. coagulans* KCTC 1805 (b1805), *B. coagulans* KCTC 1807 (b1807), *B. coagulans* KCTC 1809 (b1809), *B. coagulans* KCTC 1823 (b1823), *B. coagulans* KCTC 3825 (b3825), *B. coagulans* KFRI 841 (b841) *Sporolactobacillus inulinus* ATCC13538(SL), *Lactobacillus sporogenes* CU-815 (815) and *Bacillus polypermenticus* SCD BP-1 (BP-1).

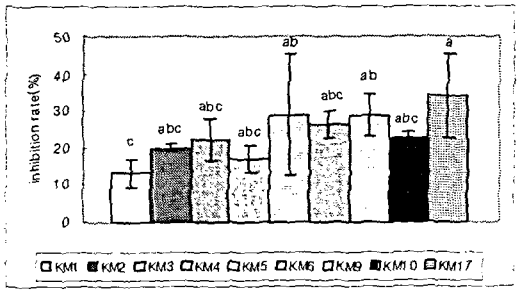

Fig. 2. Lipid peroxidation inhibitory activities of spore forming lactic acid bacteria of isolated cultures. *Bacillus megaterium*(KM1), *Bacillus circulans*(KM2), *Bacillus stearothermophilus* (KM3), *Bacillus circulans* (KM4), *Bacillus licheniformis* (KM5), *Bacillus megaterium* (KM6), *Bacillus circulans* (KM9), *Bacillus pumilus* (KM10) and *Bacillus licheniformis* (KM17).

a insignificant statistical correlation. The results on carcinoma cell proliferation inhibition by spore forming lactic acid bacteria and antioxidative activity *Bacillus coagulans* of this study. agrees with those of Kullisarr et al.(2002). And it has been suggested that strains having high level in GSH possessed high level of hydroxyl radical scavenging activity(Lin and Yen, 1999).

3. Total Cell Glutathione Contents of Spore Forming Lactic Acid Bacteria

Glutathione was detedted in all the test strains the concentration varied from 5.3 to 8.2 mol/g *Lactobacillus sporogenes* CU815 showed the highest GSH content(8.2 mol/g), *Bacillus coagulans* KCTC1805, *Bacillus coagulans* KCTC1807 had the lowest.

Total glutathione content of intracellular cell free extract of spore forming lactic acid bacteria were shown in Table 2.

Since glutathione can detoxify free radicals(Fahey et al, 1978) we hypothesized that it could represent the protection system operating against peroxy radicals .The protective role of glutathione has been stressed by Sherrill and Fahey(1988) Aerobic growth of lactic acid bacteria results in the formation of H_2O_2, O_2^- and OH compounds that are detrimental to lactic acid bacteria, previous investigators demonstrated that the addition of reduced GSH diminished peroxide production by milk grown in lactococcal cultures(Gililand and Speck, 1969).

Beneficial activities of lactic acid bacteria could be explained on the basis of the boosting effect of cell glutathione, an antioxidant in cell, which neutralize free radicals by handing off an electron then pairing off to remain neutral themselves. The destructive hydroxyl radicals is changed to H_2O by getting electron from glutathione sulfhydroxyl molecule (Anderson, 1985).

Spearmans' rank correlation quotient between the tumor cell proliferation inhibition and cellular GSH levels was shown to be 0.18, which means an insignificant positive correlation.

Table 2. Total cell glutathione contents of spore forming lactic acid bacteria

Spieces	Total GSH(mol/g)	SEM
Bacillus coagulans KCTC1015	6.30c	0.81
Bacillus coagulans KCTC1805	5.34d	0.79
Bacillus coagulans KCTC1807	5.34d	0.79
Bacillus coagulans KCTC1809	5.68d	0.79
Bacillus coagulans KCTC1823	6.43c	0.81
Bacillus coagulans KCTC3625	6.59c	0.81
Bacillus coagulans KFRI 841	6.46c	0.81
Bacillus coagulans CU819	7.71a	0.83
Bacillus polypermenticus SCD BP-1	7.26b	0.83
Lactobacillus sporogenes CU815	8.19a	0.84
Sporolactobaciiilus inulimus ATCC13538	7.87a	0.84

Ⅳ. Abstract

Tumor cell proliferation inhibitory, antioxidative activities and glutathione content were analyzed in a variety of spore forming lactic acid bacteria. Tumor cell proliferation inhibitory activity varied widely depending upon the strains of spore forming lactic acid bacteria and the types of carcinoma cell lines(0～56.7%), *Bacillus coagulans* KTCC3625 has shown a marked antiproliferative effect against the carcinoma cells and NCL-H1299 human lymphoma cell line tended to be least affected by the spore forming lactic acid bacterial cell extracts. Antioxidative activity analyzed in the lipid peroxidation occurred in all the test strains varied on the strains(5.0 to 52.0%) an extensively high degree of antioxidative activity was demonstrated by three strains of *Bacillus coagulans* KTCC3625, *Bacillus coagulans* KTCC1015 and *Lactobacillus sporogens* CU 815. Concentrations of glutathione were highest in a strain of *Lactobacillus sporogenes* CU 815 followed by *Sporolactobaciiilus inulinus* ATCC13538 (5.34 to 8.19 mol/g).

Spearmans' rank correlation quotient between cellular GSH levels and linoleic acid peroxidation inhibitory effects of the spore forming lactic acid bacteria revealed highly significant correlation quotient of 0.78. Spearmans' rank correlation quotient between the Caski human cervix carcinoma cell proliferation inhibitory activity and the linoleic acid peroxidation inhibitory effects of the spore forming lactic acid bacteria and that between Caski carcinoma cell proliferation inhibitory activity and the cellular GSH levels were shown to be 0.29 and 0.32, respectively, which means an insignificant positive correlation however.

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