In vitro Antioxidative Properties of Lactobacilli

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ABSTRACT : The antioxidative properties of lactobacilli originating from humans (*Lactobacillus acidophilus* KCTC 3111, *Lactobacillus jonsonii* KCTC 3141, *Lactobacillus acidophilus* KCTC 3151, and *Lactobacillus brevis* KCTC 3498) were investigated using *in vitro* methods, including inhibition of lipid peroxidation, resistance to hydrogen peroxide and hydroxyl radical, hydroxyl radical scavenging activity, and glutathione peroxidase (GPX) activity. *L. acidophilus* KCTC 3111 showed the highest inhibition of lipid peroxidation in both intact cells (49.7%) and cell lysate (65.2%). This strain exhibited resistance to hydrogen peroxide and hydroxyl radical, which was viable for 7 h in the concentration of 1.0 mM hydrogen peroxide. In addition, this strain showed high hydroxyl radical scavenging activity. In the GPX activity assay, the highest activity was measured in *L. brevis* 3498. GPX activity of *L acidophilus* 3111 was lower than that of *L. brevis* 3498. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 2 : 262-265)***

Key Words : *Lactobacillus*, Lipid Peroxidation, Free Radicals

INTRODUCTION

Oxidative stress occurs when the available supply of the body's antioxidant is insufficient to handle and neutralize free radicals (Halliwell and Gutteridge, 1989). Free radicals are highly unstable molecules that interact with other molecules in our bodies to destroy cellular membranes; enzymes and DNA. They accelerate aging and contribute to the development of many diseases, including cancer and heart disease (Battino et al., 1999).

Antioxidants are chemical compounds that can prevent, stop, or reduce oxidative damage. Antioxidants can protect the human body from free radicals and retard the progress of many diseases. Therefore, the development and utilization of effective antioxidants are desired (Kinsella et al., 1993; Moure et al., 1996; Lai et al., 2001).

Microorganisms have antioxidative systems to maintain free radicals levels that are not toxic to the cells (Farr and Kogoma, 1991). Recently, there has been an interest in the antioxidant effects of microorganisms and their role in health and disease. Several investigations were conducted to study antioxidative properties of lactic acid bacteria *in vitro* and *in vivo* (Kaizu et al., 1993; Ahotupa et al., 1996; Korpela et al., 1997; Lin and Yen, 1999a-c; Stecchini et al., 2001; Ito et al., 2003).

Lactobacillus spp. indigenous to humans is more likely to survive in the human intestinal tract than non-human strains, and must be able to adhere to the lining of the intestinal tract to exert their greatest benefits into the gut (Golden and Gorbach, 1992; Salminen and Wright, 1993). *Lactobacillus* with antioxidant activity may be used to help the human body by reducing oxidative damage.

The objective of this study was to test *in vitro* antioxidative properties of lactobacilli originating from humans.

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MATERIALS AND METHOD

Bacterial strains

All the strains used in this study were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). *L. acidophilus* KCTC 3111, *L. acidophilus* KCTC 3151, and *L. brevis* KCTC 3498 originated from human feces, and *L. jonsonii* KCTC 3141 originated from human intestine. The strains were cultivated at 37°C for 48 h anaerobically (BBL gas pack anaerobic system) in MRS broth (Difco). All strains were serially transferred at least three times prior to use.

Preparation of cell lysate and intact cells

Cells were harvested by centrifugation at 4°C for 30 min (5,000×g) after overnight incubation at 37°C and the pellet was washed twice with 20 mM sodium phosphate buffer (SPB, pH 7.4) and then re-suspended in SPB. Washed cell suspension was disrupted with an ultrasonic cell disrupter (Brandson Sonic Power, USA) in ice bath. Cell debris was removed by centrifugation (10,000×g for 10 min at 4°C) and filtration (0.45 μm, Millipore). Protein concentration was measured by the Bradford method (Bio-Rad Laboratories), and adjusted to 1 mg/ml.

For the preparation of intact cells, cells were washed twice with SPB and resuspended in SPB. The total cell number was adjusted to 10^9 cfu/ml.

Inhibition of lipid peroxidation

The inhibition of lipid peroxidation was determined according to the TBA (thiobarbituric acid) method (Lin and Chang, 2000) and described in our previous study (Kim et

Table 1. Inhibition of lipid peroxidation of the lactobacilli

Strains	Inhibition of lipid peroxidation (%)	
	Cell lysate	Intact cells
L acidophilus KCTC 3111	65.2±5.3[a]	49.7±1.0[a]
L. jonsonii KCTC 3141	7.1±3.1[c]	ND [b]
L. acidophilus KCTC 3151	21.6±6.3[b]	ND [b]
L. brevis KCTC 3498	22.8±0[b]	ND [b]

Total cell numbers were adjusted to 10^9 cfu/ml for the intact cells and to one mg protein/ml for cell lysate.

[a-c] Values in the same column with different superscripts letters are significantly different ($p<0.05$). ND: Not detected.

al., 2005). Briefly, reaction mixtures contained 0.6 ml of 20 mM SPB, 1 ml linoleic acid emulsion (0.1 ml linoleic acid; 99%, Sigma, 0.2 ml Tween 20 and 19.7 ml deionized water) and 0.2 ml of sample. Lipid peroxidation was started by addition of $FeSO_4$ (0.2 ml, 0.01%) and H_2O_2 (0.2 ml, 0.56 mM). The reaction mixture was incubated at 37°C for 6 h. Trichloroacetic acid (TCA; 0.2 ml, 4%), TBA (2 ml, 0.8%) and butylated hydroxytoluene (BHT; 0.2 ml, 0.4%) were then added, and the mixture was boiled at 100°C for 20 min. At room temperature, the amount of lipid peroxidation was determined by measuring the absorbance at 532 nm using Jasco V530 spectrophotometer (Japan), and expressed as the percentage of inhibition of lipid peroxidation with a control containing no sample.

Resistance to hydrogen peroxide and hydroxyl radical

The resistance of lactobacilli in the presence of hydrogen peroxide and hydroxyl radical was observed by the method of Kullisaar et al. (2002). For the measurement of resistance to hydrogen peroxide, cells were suspended at the level of 10^7 cfu/ml in SPB and incubated with 1.0 mM hydrogen peroxide (30%, Wako) at 37°C. At 1 h time intervals, the number of viable cells was estimated on MRS agar plates.

For resistance to hydroxyl radical, cells (10^7 cfu/ml) were incubated with the solution containing 10 mM THA (terephthalic acid, Sigma) in SPB and 0.01 mM $CuSO_4 \times 5H_2O$. The reaction was started by the addition of 1mM hydrogen peroxide, and the number of viable cells was estimated as indicated above.

Hydroxyl radicals scavenging activity

The hydroxyl radical scavenging activity was determined by the method of Kullisaar et al. (2002). To the 2 ml of 10 mM THA solution in SPB was added the 0.1 ml of sample. Then the hydroxyl radicals were generated via the Fenton reaction by addition of 0.1 ml $CuSO_4 \times 5H_2O$ and 0.1 ml hydrogen peroxide (the final concentration of the latter was 0.01 mM). The reaction product of THA with hydroxyl radicals (THA-adduct) was detected by using fluorescence spectrophotometer (Bio-TEK instruments SMF25, Switzerland) at 312 nm excitation and 426 nm emission. The hydroxyl radical scavenging activity was expressed as the inhibition percentage of generation of THA-adduct by sample.

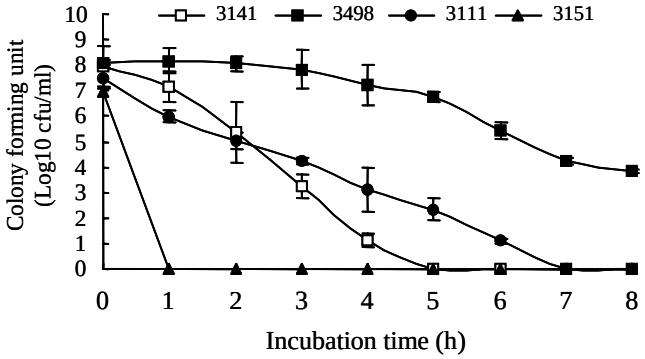

Figure 1. The survival of the lactobacilli in the presence of hydrogen peroxide. Strains were suspended at the level of 10^7 cfu/ml and incubated with 1.0 mM hydrogen peroxide. 3111: *L. acidophilus* KCTC 3111, 3141: *L. jonsonii* KCTC 3141, 3151: *L. acidophilus* KCTC 3151, 3498: *L. brevis* KCTC 3498.

Glutathione peroxidase (GPX) activity

GPX activity was determined by using a commercially available kit (RANSEL, Randox Laboratories, UK). GPX catalyzes the oxidation of GSH to GSSG. The rate of GSSG formation was measured by the decrease in absorbance with the reaction mixture containing NADPH and glutathione reductase (GR) at 340 nm. The 50 μl of cell lysate was mixed with 1 ml reagent and 40 μl of cumene hydroperoxide, and incubated at 37°C for 1 min. The kinetics were followed for 1 min at 340 nm. GPX activity (U) was expressed as nmole NADPH oxidized/min/mg protein.

Statistical analysis

The values were expressed as means±SD. Statistical analysis was performed by one-way analysis of variation (ANOVA), and significant differences were detected ($p<0.05$) by Duncan's multiple range tests using a PC statistical package (SAS, release 8.01, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Inhibition of lipid peroxidation

Inhibition of lipid peroxidation of lactobacilli is shown in Table 1. Of all the *Lactobacillus* examined in this study, *L. acidophilus* 3111 exhibited the highest inhibition of lipid peroxidation. This strain was able to inhibit lipid peroxidation by 65.2% in cell lysate and 49.7% in intact cells, while *L. acidophilus* 3141 showed little inhibition of peroxidation in both cell lysate and intact cells.

Lipids are major targets during oxidative stress. Free

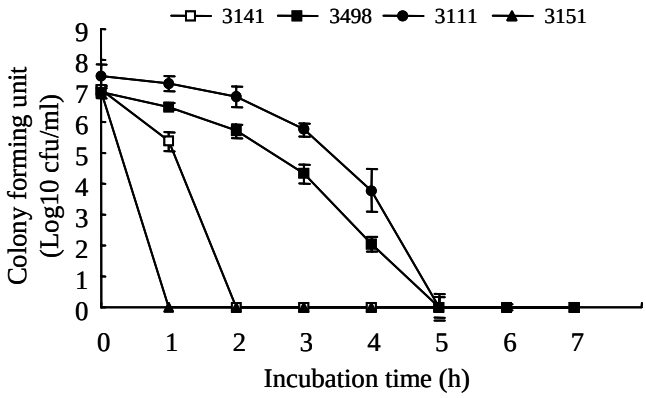

Figure 2. The survival of the lactobacilli in the presence of hydroxyl radical. Strains were suspended at the level of 10^7 cfu/ml and incubated with hydroxyl radical. Hydroxyl radicals were generated via the Fenton reaction. 3111: *L. acidophilus* KCTC 3111, 3141: *L. jonsonii* KCTC 3141, 3151: *L. acidophilus* KCTC 3151, 3498: *L. brevis* KCTC 3498.

radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation (Aruoma, 1998). Effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. Therefore, lipid peroxidation protection is essential (Halliwell and Gutteridge, 1989).

Investigations by Lin and Chang (2000) have revealed that intestinal *L. acidophilus* ATCC 4356 inhibited lipid peroxidation by 45.3% in cell lysate and 28.1% in intact cells. Also, Kullisaar et al. (2002) reported that fecal *L. fermentum* E-3 showed good inhibition of lipid peroxiation by 59% in cell lysate and 21% in intact cells.

Resistance to hydrogen peroxide and hydroxyl radical

We examined the resistance of lactobacilli to hydrogen peroxide and hydroxyl radicals. As shown in Figure 1, *L. acidophilus* 3111 was viable for 7 h in the concentration of 1.0mM hydrogen peroxide while *L. acidophilus* 3151 survived only for 1 h. In the presence of highly damaging hydroxyl radicals, *L. acidophilus* 3111 and *L. brevis* 3498 survived for 5 h, but *L. acidophilus* 3151 survived only for 1 h (Figure 2).

Antioxidative activity of microorganisms is one of the reasons for their increased resistance to reactive oxygen species (ROS). Kullisaar et al. (2002) reported that the antioxidant strains have significantly high resistance to ROS compared with the low-antioxidant strains. However, in this experiment, the inhibition of lipid peroxidation was not similar to that of the range of resistance to ROS. Although *L. brevis* 3498 had a very low lipid peroxidation inhibition, it survived for 8 h. The 1.0 mM hydrogen peroxide did not have a direct effect on viability of *L. brevis* 3498.

Table 2. Hydroxyl radical scavenging activity of the lactobacilli

Strains	Hydroxyl radical scavenging activity (%)	
	Cell lysate	Intact cells
L acidophilus KCTC 3111	70.0±4.9[a]	53.0±6.5[a]
L. jonsonii KCTC 3141	32.8±5.2[b]	22.6±2.9[b]
L. acidophilus KCTC 3151	27.3±2.8[b]	13.5±3.2[b]
L. brevis KCTC 3498	18.9±4.7[c]	0.9±0.4[c]

Total cell numbers were adjusted to 10^9 cfu/ml for the intact cells and one mg protein/ml for cell lysate.
[a-c] Values in the same column with different superscripts letters are significantly different ($p<0.05$).

Table 3. Glutathione peroxidase (GPX) activity of the lactobacilli

Strains	GPX (U/mg protein)
L acidophilus KCTC 3111	10.5±0.8[b]
L. jonsonii KCTC 3141	ND[d]
L. acidophilus KCTC 3151	3.9±0.0[c]
L. brevis KCTC 3498	25.6±0.1[a]

[a-d] Values in the same column with different superscripts letters are significantly different ($p<0.05$).
ND: Not detected.

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells because it may give rise to hydroxyl radicals (Deutsch, 1998). Many proteins are known to be sensitive to physiological levels of hydrogen peroxide as low as 100 nM. In *Escherichia coli*, concentrations of hydrogen peroxide between 1 and 3 mM resulted in cell death, causing DNA damage (Imlay and Linn, 1986).

Hydroxyl radical scavenging activity

Table 2 showed that lactobacilli had various hydroxyl radical scavenging activities. *L. acidophilus* 3111 was the most active one among the strains, for which hydroxyl radical scavenging activity was 70.0% in cell lysate and 53.0% in intact cells. But other strains showed low activity in both cell lysate and intact cells.

Antioxidative property, especially radical scavenging activity, is very important due to the deleterious role of free radicals. The hydroxyl radicals are extremely reactive free radicals formed in biological systems. Hydroxyl radicals participate in the initiation of lipid peroxidation. They react rapidly with almost every type of molecule in living cells, such as amino acids, phospholipids, DNA, bases, and organic acids. It has been reported that scavenging of different types of ROS was thought to be one of the main antioxidant mechanisms of the antioxidant action exhibited by lactic acid bacteria (Namiki, 1990). Hydroxyl radical scavenging of *Lactobacillus* seems to be an endogenous property and it is not regulated by the changes in the environmental conditions or the imposed oxidative stress, which were similar in cells exposed to anaerobic and high oxygen conditions (Amanaditou et al., 2001).

GPX activity

In the GPX activity assay, the highest activity was measured in *L. brevis* 3498 (Table 3). *L acidophilus* 3111 had the GPX activity for 10.5 U/mg protein. Not all strains tested in this study exhibited glutathione reductase (GR) activity (data not shown).

ROS can be detoxified in microorganisms by a number of antioxidant enzymes, GPX and GR are two important antioxidant enzymes, which protect cells from oxidative damage by scavenging ROS and maintaining the level of glutathione. Cell viability is more drastically affected when GPX is inhibited. Increasing the level of this enzyme results in higher resistance of the cell toward oxidative stress (Tabatabaie and Floyd, 1994).

CONCLUSION

In this study, we tested the antioxidative properties of lactobacilli originated from humans using *in vitro* methods. From the results obtained, *L. acidophilus* 3111 exhibited the highest inhibition of lipid peroxidation in both cell lysate and intact cells. Also, this strain exhibited resistance to hydrogen peroxide and hydroxyl radicals, and was viable for 7 h in a concentration of 1.0 mM hydrogen peroxide. However, resistance to hydrogen peroxide seemed to have no relation to inhibition of lipid peroxidation since *L. brevis* 3498 with low lipid peroxidation inhibition survived for 8 h. *L. acidophilus* 3111 was the most active one among the strains for hydroxyl radical scavenging activity. In the GPX activity assay, the highest activity was measured in *L. brevis* 3498 while *L acidophilus* 3111 showed lower GPX activity than *L. brevis* 3498. Further experiments should be performed to obtain more evidence for the antioxidant activity of *Lactobacillus*.

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