Occurrence of Glutathione Sulphydryl (GSH) and Antioxidant Activities in Probiotic *Lactobacillus spp.**

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ABSTRACT : The antioxidative ability on the basis of reduced glutathione sulphydryl level, the inhibition activities of linoleic acid peroxidation of cell free extract of *Lactobacillus spp.* and the effects of types of media and growth phase of the cells on the cellular GSH level have been determined. Correlation between reduced glutathione sulphydryl level and antioxidative ability of *Lactobacillus spp.* was analyzed: *Lactobacillus casei* HY 2782 contained 25.15 μmole/g of GSH, the cellular GSH level of *L. casei* HY 2782 reached maximum after 24 h of cultivation and tended to decrease on further cultivation up to 72 h. There was a significantly higher level of cellular GSH when grown in de Man Rogosa and Sharpe (MRS) broth than in tryptone phytone yeast extract (TPY) broth or bromcresol pruple dextrose (BCP) broth ($p<0.05$). The antioxidant activity of cell free extract of *Lactobacillus spp.* have been shown to be significantly different among strains in the inhibition of linoleic acid peroxidation by thiobarbituric acid (TBA) test ($p<0.01$). *L. casei* HY 2782 and *L. acidophilus* ATCC 4356 revealed a high degree of antioxidative effect in linoleic acid oxidation system. Spearmans' rank correlation coefficient between inhibitory activity on linoleic acid peroxidation and cellular GSH levels of *Lactobacillus spp.* was 0.65, which means a significant positive correlation. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 11 : 1582-1585)***

Key Words : GSH Level, Antioxidant Effect

INTRODUCTION

During past decade, oxidative stress and antioxidative potency have been revealed as the key points in molecular regulation of cellular stress responses (Demple et al., 1999). Oxidative stress occurs when abnormally high levels of reactive oxygen species are generated, resulting in DNA, protein and lipid damage. The important components of the cellular defense system are reduced glutathione (GSH) and antioxidative enzymes. Reduced glutathione (γ-glutamylcysteinylglycine) has long been thought to occur in all living cells, and it has been known that sulfur containing amino acids cysteine, glutamic acid and glycine are constituents synthesizing glutathione molecule whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells which functions as intracellular reductant and plays important roles in catalysis, metabolism and transport (Rose, 1980). It protects cells against free radicals, reactive oxygen species (ROS) and toxic compounds of endogenous and exogenous origin (Rose, 1980; Anderson, 1985; Bounous, 1989; Bounous and Gold, 1991; Yihong et al., 1998). Many published reports concern the relationship between cellular glutathione antioxidant and age-related human disorders. Peroxidants accumulated in human body were reported to cause disorders, and oxidative stress plays an important pathological role in human diseases, cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative damage (Halliwell and Gutteridge, 1984; Bounous et al., 1991). Those disorders were named 'free radical disease' by Harman (1984). The human body has an inherently antioxidative system ie. superpxode dismutase, glutathione peroxidase and uric acid to protect itself from damage caused by peroxidants (Frei et al., 1988; Frei et al., 1989).

Lactobacillus acidophilus, L. casei and *Bifidobacterium spp.* have attracted a lot of attention among lactic acid bacteria for their potential role in promoting human health. *Lactobacillus spp.* are utilized for the production of fermented dairy products which have been consumed for centuries and are becoming popular due to their beneficial effects on human health. The potential health attributes of Lactobacilli include improvement of lactose intolerance, control of gastrointestinal infections, reduction of serum cholesterol, stimulation of immunological system and anticarcinogenic actions (Perdigon et al., 1990; Tammy et al., 1994; Yoon and Won, 2002; Byun et al., 2003). It has been shown that some Lactobacilli possess antioxidative activity, and are able to decrease the risk of accumulation of ROS during ingestion of food, lactic acid bacteria are able to degrade the superoxide anion and hydrogen peroxide (Ahotupa et al., 1996). The antioxidative effect of lactic acid bacteria has been reported only recently, there is not much data about the antioxidative abitlity of lactic acid bacteria available (Ahotupa et al., 1996).

This study was aimed for finding out glutathione levels of probiotic *Lactobacillus spp.*, the effects of media and

* This Research was supported by the Chung-Ang University Research Grants in 2003.

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Received February 4, 2004; Accepted June 9, 2004

growth phase on the cellular glutathione of the selected strain .and for the elucidation of the antioxidant activity of cell free extract of *Lactobacillus* in the linoleic acid peroxidation system by thiobarbituric acid (TBA) assay.

MATERIALS AND METHODS

Bacterial strains and media

A total of twenty two probiotic strains were used in this study; nineteen strains of *Lactobacillus spp.* strains were cultured in MRS broth (Difco, USA) at 37°C and stored in 11% skim milk containing 0.75 M adonitol at -70°C. four strains of *Bifidobacterium spp.* were cultured in BL broth in anaerobic jar and stored in nutrient broth containing 0.75 M adonitol at -70°C. For the comparison of the media effect, tryptone phytone yeast extract (TPY, Difco, USA) broth and bromcresol pruple destroxe (BCP Difco, USA) broth were used.

Preparation of intracellular cell-free extract

Cells were cultured in appropriate medium at optimal cultivation temperature as indicated in the bacterial strains for 24 h. the culture were centrifuged at 10,000×g for 10 min 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.

Measurement of glutathione sulphydryl content in intracellular cell free extract and the effect of growth phase and media composition on GSH level of *L. casei*

Measurement of glutathione sulphydryl content in intracellular cell free extract of probiotic strains was performed using the GSH assay kit (calbiochem. Cat. No.354102. USA); took 200 μl of intracellular cell free extract and adjusted the total volume to 900 μl with buffer (solution 3) and added 50 μl of solution R1 and mixed thoroughly, and added 50 μl of solution R2 and mixed thoroughly and then incubated the sample at 25°C for 10 min in the dark and measured the final absorbance (A) at 400 nm and the calculation was performed using the following equation:

$$GSH = \frac{(A-A_O)\times D}{\varepsilon}$$

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.

Culture samples of *L. casei* HY 2782 were taken at 24 h. 48 h. and 72 h after inoculation and intracellular cell free extract was prepared and triplicate samples were analyzed for the GSH level. The effect of media composition was determined by the *L. casei* was cultivated in MRS. TPY and BCP media and the resulting cultures were determined for the level of intracellular GSH.

Determination of antioxidative activities in linoleic acid peroxidation system

Linoleic acid was chosen as a source for unsaturated fatty acid. and the analysis of the thiobarbituric acid (TBA) method was used for the measurement of lipid peroxidation. Twenty milliliters of linoleic emulsion was made up of 1 ml of linoleic acid. 0.2 ml of Tween 20. and 19.7 ml of deionized water. A sample of 0.5 ml of phosphate buffer solution (0.02 M. pH 7.4). 0.2 ml of linoleic acid emulsion. 0.2 ml of ascorbate (0.01%) and 0.4 ml of intracellular cell-free extract were mixed and incubated at 37°C. After 12 h of incubation. 1ml of the reaction solution was mixed with 0.2 ml of trichloroacetic acid (TCA 4%). 2 ml of TBA (0.8%) and 0.2 ml of butylated hydroxytoluene (BHT 0.4%). This mixture was incubated at 100°C for 30 min and allowed to cool. Two milliliters of chloroform was then added for extraction. The extract was obtained. and the absorbance was measured at 532 nm. The percentage of inhibition of linoleic acid peroxidation was defined as follows; (1-A532 (sample)/A 532 (blank)×100%.

Statistical analysis

Within the same treatment group, for the comparison of the glutathione and the percentage of inhibition of linoleic acid peroxidation values between the treatment values were compared using SAS Duncan's multiple-range test. Spearmans' rank correlation quotient were determined between inhibitory activity on linoleic acid peroxidation and cellular GSH levels (SAS 1990).

RESULTS AND DISCUSSION

Total glutathione content of intracellular cell free extract of *Lactobacillus spp.* were shown in Figure 1. All the Lactobacilli strains possessed at least 7.4 μmol/g of glutathione sulphhydryl in their cytoplasm. seventeen strains could be classified into three statistically different groups based on their cell GSH content ($p>0.01$).

L. casei HY 2782, *L. rhamnosus* CU 01 and *L. plantarum* CU 03 had the high level GSH group having more than 14.0 μmol/g of glutathione sulphhydryl, and *L. casei* HY2782 possessed 25.1 μmol/g of glutathione sulphhydryl. A moderate level of cellular GSH were present

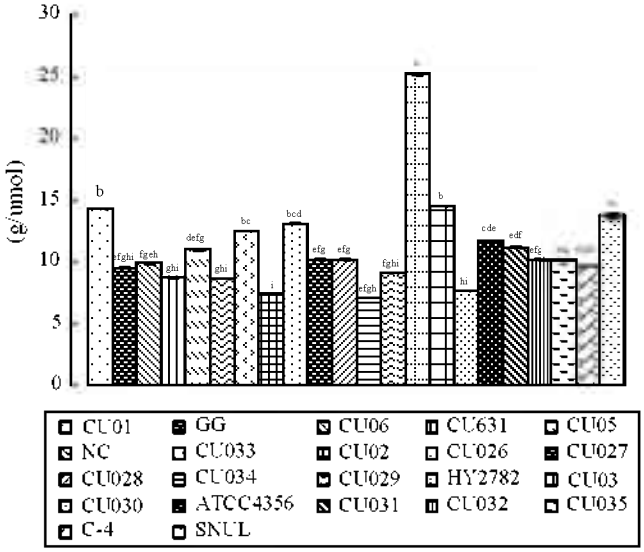

Figure 1. Total glutathione content for Intracellular free extract of *Lactobacillus* spp. and *Bifidobacterium* spp. [a-i] Values with diffrent superscripts in the same with are different ($p<0.05$). Strain designations CU01: *L. rhamnosus*, GG: *L. rhamnosus*, CU06: *L. brevis*, CU631: *L helveticus*, CU05: *L. brevis*, NC: *L. acidophilus*, CU033: *Bif. Lactis*, CU02: *L. rhamnosus*, CU026: *L casei*, CU027: *L. acidophilus*, CU028: *L. acidophilus*, CU034: *Bif. Longum*, CU029: *L. casei*, HY2782: *L. casei*, CU03: *L. plantarum*, CU030. *L. casei*, ATCC4356. *L. acidophilus*, CU031: *Bif. longum*, CU032: *Bif. Infantis*, CU035: *L. casei*, C-4: *L. Johnsoni*, SNUL: *L. acidophilus*.

in the cytoplasm of *L. casei* CU 026, *L. acidophilus* 4356 and *L. brevis* CU 05 with average content of 12.0 μmol/g of glutathione sulphhydryl. The rest strains determined were as the low GSH groups which contain less than 12.0 μmol/g.

Our previous study (Byun et al., 2004) on the effects on feeding *Lactobacillus spp.* on the levels of cell glutathione in kidney, liver and erythrocyte in ICR mice revealed significantly higher kidney and liver GSH which fed *L. casei* HY2782, further studies on the tendency of high GSH level in ICR mice by feeding HY 2782 feeding are needed. An intensive variation in GSH content of *Lactobacillus spp.* has been revealed due to the strains of Lactobacilli which agreed with the results of Kullisr et al. (2002).

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).

Many kinds of reactive oxygen species could be formed in the human body (oxidative stress), free radicals can damage or destroy cell walls causing apoptosis and disrupt DNA, then pathogenesis could be proceeded. Existence of enough antioxidants was effective for the reduction of

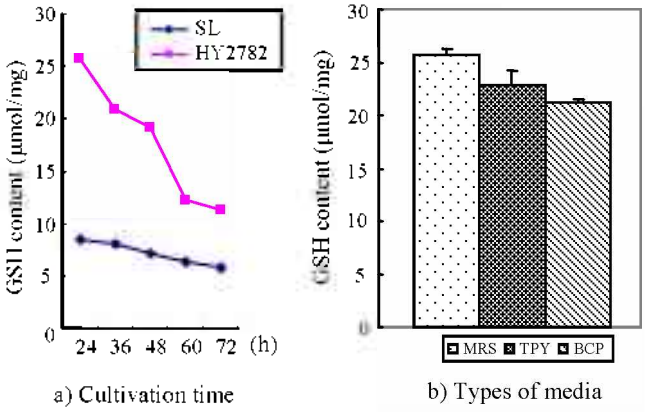

Figure 2. Effects of types of media (MRS: de Mann, Rogosa and Shrape medium, TPY: tryptone phytone yeast extract medium, BCP: bromcresol purple dextrose medium) and Growth phase on the cellular GSH in *Lactobacillus casei* (HY 2782) and *Sporolactobacillus* (SL).

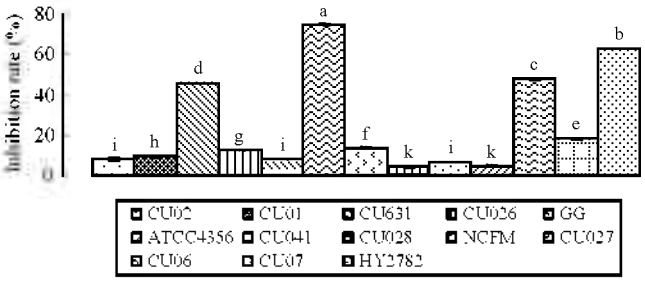

Figure 3. Inhibition of linoleic acid peroxidation by Intracelluar cell-free extraction of Lactobacilli. [a-i] Values with different superscripts in the same are different ($p<0.05$). Strain designation, CU02: *L. rhamnosus*, CU01: *L. rhamnosus*, CU631: *L. helveticus*, CU026: *L. casei*, GG: *L. rhamnosus*, ATCC4356: *L. acidophilus*, CU041: *L. salivarius*, CU028: *L. acidophilus*, NCFM: *L. acidophilus*, CU027: *L. acidophilus*, CU06: *L. brevis*, CU07: *L. fermentum*, HY2782: *L. casei*.

reactive radicals by scavenging reactive oxygen species. otherwise glutathione depletion and the subsequent low stores of protein thiol result in both calcium release from intracellular calcium and cytotoxicity (Anderson,1985; Bounous and Gold, 1991) Elevated scavenging of reactive oxygen by glutathione would be a good trait of Lactobacilli. The cellular level of GSH in Lactobacilli in this study were much higher than the results reported by Robert et al. (1987). these discrepancies could be attributed to the variations in those test strains.

Cellular GSH levels of *L. casei* HY 2782 and *Sporolactobacillus sp.* have been determined daily after inoculation, they tended to decrease as incubation proceeds and a significant difference in cellular GSH level has been revealed between *L. casei* HY 2782 and *Sporolactobacillus* sp as shown in Figure 2a).

The effects of three types of on the cellular GSH level were compared and the results showed that a significant

difference among the media types existed high in MRS, medium in TPY and low BCP. Cellular GSH levels of *L. casei* HY 2782 was affected by both cultivation time and by types of media. Difference in the media composition and types of nitrogen source could be the cause of variation in the cellular GSH level.

As shown in Figure 3, selected strains of of *Lactobacillus spp.* were utilized to determine antioxidative activity in the linoleic acid peroxidation system. all the *Lactobacillus* strains demonstrated an inhibitory effect on linoleic acid peroxidation. The inhibitory rates on linoleic acid peroxidation ranged from 5.0 to 70.0% when intracellular cell free extract was added. The results indicated that all strains tested demonstrated antioxidative activity of inhibiting lipid peroxidation. and an extensively high degree of inhibitory activity on linoleic acid peroxidation occurred by four strains of *Lactobacillus spp.*: *L acidophilus* ATCC 4356. *L casei* HY 2782, *L. brevis* CU 06 and *L. helveticus* CU 631.

It has been reported that six strains of *L. acidophilus* revealed high degree of inhibitory activity on linoleic acid peroxidation by Lin and Yen (1999). which shows some discrepancies with this study. There exists some variation among the strains of *L. acidophilus* and the other species in *Lactobacillus* showed inhibitory activity on linoleic acid peroxidation. Spearmans rank correlation between inhibitory activity on linoleic acid peroxidation and cellular GSH levels of 11 strains of *Lactobacillus spp.* analysed and the correlation quotient was 0.65 which means a significant statistical correlation. An intensive antioxidative activity manifested in the linoleic acid peroxidation could be caused by the high levlel of cellular glutathione as a scavenging power against reactive oxiygen species generated in the cells.

The results on glutathione level and antioxidative activity of *Lactobacillus spp.* of this study agree 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.

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