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Enhancement of immunological activity in exo-biopolymer from submerged culture of *Lentinus edodes* with rice bran

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Abstract – The objective of the present study was to determine the possible immune-enhancing effects of a substance extracted from a submerged culture of *Lentinus edodes* with rice bran (SLRB). According to the results obtained by measuring the *in vitro* macrophage activity of the exo-biopolymer from SLRB, it appears to exhibit activity similar to that of LPS, and this activity seems to occur in a dose-dependent manner. According to the results obtained by measuring splenocyte proliferation, the exo-biopolymer appears to induce an increase in proliferation of approximately 1.4-fold compared to the control group. We measured the proliferation of bone marrow cells in order to evaluate gut immunity and, according to our results, proliferation was increased to 109% that of the control group,and was similar to that associated with LPS. In order to characterize the enhancement of immunological activity *in vivo*, we orally administered the exo-biopolymer (25, 50, 250 mg/kg bw) to C3H/He mice, and then measured the macrophage activity, determining that the activity was higher than that of the controls at concentrations of 50 and 250 mg/kg. Therefore, the exo-biopolymer from SLRB can be considered to be a useful a BRM agent, as it clearly allows some protection against immunological diseases.
Keywords – rice bran, *Lentinus edodes,* immune stimulating activity, BRM (biological response modifiers)

Introduction

The human lifespan has obviously been greatly extended in recent times as the result of changes in eating habits and medical developments. However, as a consequence of this, the incidence of adult diseases, chronic regressive diseases, geriatric diseases, etc. are increasing, due partially to a generalized lack of exercise resulting from the progress of civilization (Hirokazu, 1994). In response to this problem, active research is currently underway to identify anticancer elements and biological response modifiers (BRM) in natural substances and functional elements in food, and to employ them in the maintenance and facilitation of health (Kim and Kim, 1997). Much of this has focused on the lactobacilli, a category of organisms which includes *Lactobacillus* and *Leuconostoc*, mushrooms such as *Agaricus blazei* and *Lentinus edodes*, ginseng, etc., all of which are known to enhance immunological activities, and all of which exert anticancer effects. Recently, the phyto-polysaccharides obtained from natural plants such as mistletoe, as well as from the aleurone layer of grains, have been identified as novel BRM agents (Hong, 2005).

Rice bran is a byproduct of the hulling of rice, an important food resource in Korea. Around 600,000 tons of rice bran is produced in Korea every year. As more sophisticated food processing technologies are developed and come into play, rice bran production is expected to increase. Also, interest in the physiological properties harbored by rice bran extracts is currently on an upward trend (Juliano, 1985). In rice bran, certain chemical elements and arabinoxylan (Ooi and Liu, 2000; Tzianabos *et al.*, 2003), a polysaccharide which enhances immunological activity, are known to activate NK cells, which are immunological cells which comprise part of the body's front line of defense against cancer (Ghoneum, 1999; William, 1998; Ghoneum, 1998). The active elements in rice bran exhibit truly excellent physiological activity but, because they exist in a mixture with dietary fiber, rice bran itself has very low functionality with regard to the enhancement or augmentation of immunity. Thus, a variety of techniques have been developed for the separation

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of the active elements in rice bran from dietary fiber (Andrewartha *et al.*, 1979; Henry, 1987; Williamson *et al.*, 1996). One such method involves the production of a fermented form of rice bran, using mushrooms or the carbohydrolase obtained from mushrooms (Hong, 2005). The mushroom used in this production technique is a fungus belonging to the Basidiomycota, and has also been extensively used as both a food and as a medicine. Research is currently underway regarding the anticancer, antibacterial, antihypertensive, antiviral and anticholesterol effects of this mushroom (Lee and Park, 1998; Ikekawa *et al.*, 1969; Takuma and Nobuo, 1976; Park *et al.*, 1998). Among its active elements, -1,3 glucan, a polysaccharide found in *Lentinus edodes*, is known to exert an indirect anticancer effect by augmenting immunological activity in the body. It has been reported that this effect originates from an immunity enhancement mechanism, such as the activation of the body's complement system (Denner and Tucker, 1973), the activation of macrophages (Hamuro and Wagner, 1978) or the promotion of interferon generation (Chihara, 1985). Thus, the purpose of the present study was to clearly delineate the possible immune-enhancing effects of a substance extracted (exo-biopolymer) from rice bran, using the mycelia of *Lentinus edodes*.

Experimental

Preparation of exo-biopolymer from submerged culture of *Lentinus edodes* with rice bran (SLRB) – The fungus (*Lentinus edodes)*and rice bran were both obtained from Kyonggi-do, Suwon, Korea. The exo-biopolymer from the submerged *Lentinus edodes* culture with the rice bran was lyophilized for use in the immune response assay.

Animals – ICR mice (male, 6-8 weeks old) and C3H/HeN mice (female, 6-8 weeks old) were obtained from Daehan-Biolink Co. (Chungcheongbuk-do, Korea), and were maintained on standard rodent chow and water, which was provided *ad libitum*.

Macrophage stimulation assay – Six- to eight-week old ICR male mice were interperitoneally injected with 1 ml of 3% thioglycolate medium. After 3 days, macrophage cells were obtained from the peritoneal cavities of the mice, by washing them twice with 5 ml of cold RPMI 1640 medium supplemented with 5mM HEPES, penicillin (100 U/ml), and streptomycin (100 ug/ml). An aliquot (200 ul) of the cell suspension (1×10^6 cells/ml) was then seeded in a flat-bottomed 96-well microplate. After 2 hours of incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, the non-adherent cells were removed via two washings with RPMI-1640 medium. The adherent macrophage monolayer was then used in the following experiments.Adherent macrophage cells were cultured for 24 hours in the presence of the test samples in a 96-well microplate. The macrophage monolayer, in a 96-well microtiter plate, (1×10^5 cells/ml) was solubilized via the addition of 25 ul of 0.1% Triton X-100. 150 microliters of 10.0 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to the reaction mixture, after which the absorbance was photometrically determined at 405nm, using a microplate reader (Burstone and Kalpow, 1964).

Splenocyte proliferation assay – The spleens were aseptically removed from an ICR mouse (male, 6-8 weeks old) and placed in ice-cold RPMI-1640 medium. Each of the spleens was squeezed with a 5 ml syringe plunger in order to extrude the cells. The cell suspensions were washed three times with RPMI-1640 medium. The pelleted cells were resuspended in 5 ml of 0.2% NaCl, then incubated for 5 minutes at room temperature in order to lyse the red blood cells. The splenocytes were suspended in RPMI-1640 containing 10% FBS. 90 microliters of suspended cells (5×10^6 cells/ml) were cultured with 10ul of the appropriately diluted test sample in a 96-well microplate for 3 days at 37°C, in a humidified atmosphere containing 5% CO_2 and 95% air. Then, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (1 mg/ml) was added to the sample, and the cells were incubated for an additional 5 hours. The supernatants were removed and the formazan crystals were dissolved in 100 ul of 0.04N HCl/isopropanol. Finally, the optical density was determined at a wavelength of 570 nm (Tubaro *et al.*, 1996).

Assay of intestinal immune system modulating activity – Intestinal immune system modulation was measured with Peyer's patches, in accordance with the procedure established by Hong *et al.* (1998). Peyer's patch cells were isolated from the small intestines of C3H/He mice (female, 6-8 weeks old) and suspended in RPMI1640 medium supplemented with 5% FBS. Approximately 180 ul of the cell suspension (2×10^6 cells/ml RPMI 1640-FBS) was then cultured with 20ul of the appropriately diluted test sample in a flat-bottomed 96-well microtiter plate for 5 days at 37°C, in a humidified atmosphere containing 5% CO_2 and 95% air. The resulting culture supernatant was then added to the bone marrow cell suspensions (2.5×10^5 cells/ml RPMI 1640-FBS) from the C3H/He mice, and incubated for 6 days in a humidified atmosphere containing 5% CO_2 and 95% air, in order to evaluate the growth capacity of the bone marrow cells.

After 20 ul of Alamar Blue™ (Serotec, Oxfordshire, UK) solution was added into each of the wells in a flat-bottomed microtiter plate, the cells were continuously cultured for 5-24 hours in order to conduct a cell (Page *et al.*, 1993). The cells were counted by fluorescence intensity measured during cultivation using a SPECTRAFluor Plus system (TECAN Co. Ltd., Austria) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The modulating activity of the intestinal immune system was expressed as the ratio of the growth of the bone marrow cells with the Peyer's patch cells incubated using the extract to the growth of the control cells, in which the Peyer's patch cells had been incubated with saline rather than with the sample.

Assay of macrophage stimulation *in vivo* – The samples were orally administered once a day for a total of 5 days, after which we harvested the peritoneal macrophages from the peritoneal cavities of the C3H/HeN mice. Macrophage activity-stimulatory properties were then assessed via a cellular lysosomal enzyme assay, which was based on the activity of acid phosphatase from the macrophages.

Results and Discussion

Macrophage activity-stimulating effects of the exo-biopolymer from SLRB – Immune responses can be influenced by exposure to several types of foreign substances. In the immune response, macrophages perform critical functions. They not only function as a link between the innate and acquired immune systems, but also fight infection and inflammation, and promote the healing of wounds (Sunderkotter *et al.*,4). Macrophages kill microorganisms, tumor cells,and damaged tissues during inflammation via two oxidative pathways, which involve the synthesis of superoxide anion (O^{2-}) and nitric oxide (NO) by NADPH oxidase and nitric oxide synthases (iNOS), respectively (Morel *et al.*, 1991). These cells are also capable of generating a variety of cytokines, including interleukin(IL), interferon(IFN),and tumor necrosis factor (TNF), as well as a host of active substances, most notably prostaglandin (Ramesh *et al.*, 2002).

In order to characterize the effectsof the exo-biopolymer from SLRB on macrophage activity, adherent macrophage cells were cultured in the presence of the test samples (10, 100 ug/ml) in a 96-well microplate for 24 hours. As a result, the exo-biopolymer obtained from the SLRB exhibited an activity level as high as that of LPS, depending on both the positive control used and the dosage (Fig. 1). This indicates that the exo-biopolymer

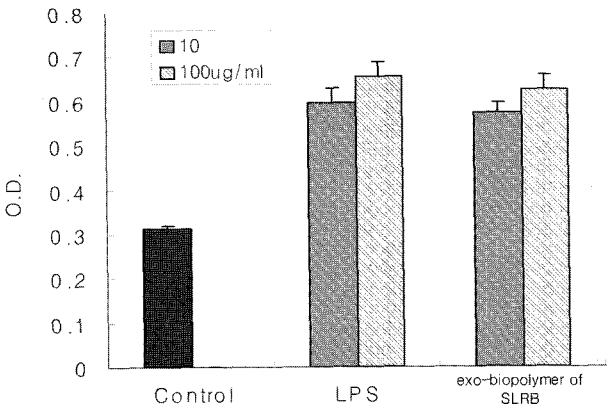

Fig. 1. Effects of exo-biopoymer from submerged culture of *Lentinus edodes* with rice bran (SLRB) on the activity of macrophage Macrophage stimulating activity is expressed as the stimulation of cellular lysosomal enzymes compared with that of the control. Values represent mean ± S.E.. LPS (lipopolysaccharide), the positive control.

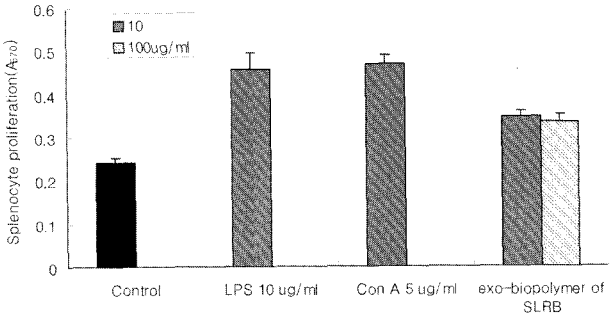

Fig. 2. Effects of exo-biopoymer from submerged culture of *Lentinus edodes* with rice bran on the proliferation of splenocytes The proliferation of splenocytes is expressed as the stimulation of cell growth of spleen cells to that of control. Values represent mean ± S.E.. LPS (lipopolysaccharide) and ConA (concanavalin A), both of them are the positive controls.

from SRLB might prove to be effective at killing tumor cells/microorganisms in both innate and adaptive immune responses, and this substance might also represent an immunopotentiator and a biological response modifier.

Splenocyte proliferation effects in the exo-biopolymer isolated from SLRB – In order to determine the effects of the exo-biopolymer from SLRB on splenocyte proliferation, we cultured splenocytes in the presence of the test samples (10, 100 ug/ml) in a 96-well microplate for 72 hours. Splenocyte proliferation was observed to have become elevated in the cultures to which the exo-biopolymer from SLRB had been added, and the cultures which had been exposed to 10 or 100 ug/ml of the exo-biopolymer exhibited a higher level of activity than was measured in the control sample (1.44, 1.39-fold control levels) (Fig. 2). However, the exo-biopolymer exerted a

less pronounced effect on splenocyte proliferation than did T cell mitogen concanavalin A (Con A; Sigma, St. Louis, MO) and B cell mitogen lipopolysaccharide (LPS; Sigma). Chae *et al.* (2004) previously evaluated the effects of arabinoxylan obtained from rice bran on splenocyte proliferation in mice, and determined that it induced a proliferating response, in a dose-dependent manner. Choi *et al.* (2002) also reported, in their research involving arabinoxylan obtained from wheat, that proliferation responses were high at high molecular weights. Accordingly, it can be surmised that the physiologically active components of fermented rice bran extract, including arabinoxylan, exert positive effects with regard to splenocyte proliferation.

Intestinal immune system activity-modulating effects of the exo-biopolymer from SLRB, determined with Peyer's patch cells – The effects of the exo-biopolymer from SLRB on the Peyer's patch cell-associated mediation of the hematopoietic responses of bone marrow cells were investigated via the measurement of *in vitro* immune response. Peyer's patch cells were obtained from C3H/He mice, and the supernatant, cultured with the samples, was added to a culture of bone marrow cells isolated from mice. We then conducted a Peyer's patch cell comparison of intestinal immune system-modulating effects, and determined that the exo-biopolymer isolated from SLRB exhibited profound activity (109% of the saline control), similar to that of the positive control and of the LPS sample (Fig. 3). This indicates that the exo-biopolymer from SLRB enhances the bone marrow cell proliferation stimulatory responses through the Peyer's patch cells. Peyer's patches are rapidly eliminated from the mucosa, migrating through the mesenteric lymph nodes until they reach the systemic circulation. The intestinal immune system, including the Peyer's patches, not only contributes to the defense system of the mucosa, but also regulates systemic inflammation, suppressing both allergic reactions and autoimmune diseases (James and Zeitz, 1994). Therefore, the exo-biopolymer isolated from the submerged culture of *Lentinus edodes* with rice bran may exert its clinical effects via the intestinal immune system.

Effects of macrophage stimulation of orally administered exo-biopolymer from SLRB – Oral administration of 50 and 250 mg/kg BW (body weight)/day of the exo-biopolymer obtained from the submerged culture of *Lentinus edodes* with rice bran for 5 days revealed a more profound stimulation of macrophage lysosomal enzyme activity (1.41,1.44-folds of control) than was observed in conjunction with a dosage of 25mg/kg BW/day (1.1-fold) (Fig. 4). This indicates that the oral

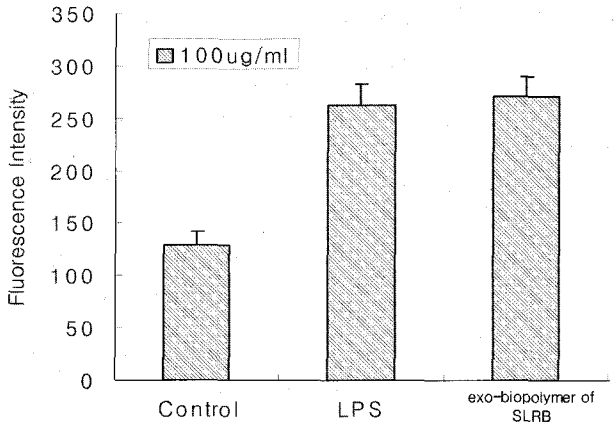

Fig. 3. Effects of exo-biopoymer from submerged culture of *Lentinus edodes* with rice bran on intestinal immune system modulating activity Intestinal immune system modulating effect is expressed as the stimulation of cell growth of bone marrow cells compared with that of the control. Values represent mean ± S.E..

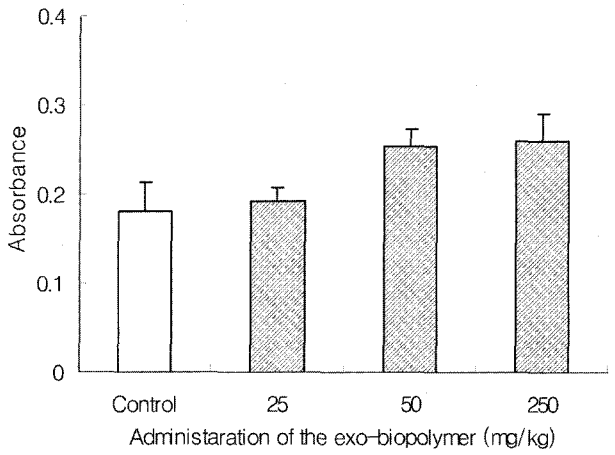

Fig. 4. Effects of exo-biopoymer from submerged culture of *Lentinus edodes* with rice bran on macrophage stimulation on oral administration Exo-biopoymer from SLRB fraction was orally administered for 5 days into C3H/HeN mice (n = 6) by 25 mg/kg, 50 mg/kg and 250 mg/kg. Data are expressed as mean ± S.E.. Control was saline only.

administration of this exo-biopolymer from SLRB enhances macrophage stimulatory responses.

In conclusion, the exo-biopolymer from SLRB increases macrophage stimulation both *in vivo* and *in vitro,* and also fosters increased splenocytic proliferation, as well as enhancing bone marrow cell proliferation-stimulatory responses. Therefore, the results of this study suggest that substances extracted from *L.edodes* with rice bran play crucial roles in improving immunoregulation. Further studies will be necessary in order to elucidate more clearly the structure of this exo-biopolymer from SLRB, as well as its relevant biochemical mechanisms.

Acknowledgment

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