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Macrophage Stimulating Activity of Exo-Biopolymer from Submerged Culture of *Lentinus edodes* with Rice Bran

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Abstract To find a new utilization of rice bran, nine higher fungi were examined for the production of exo-biopolymer with macrophage stimulating activity from rice bran. Among the exo-biopolymers produced from submerged cultures, *Lentinus edodes* showed the highest activity, followed by *Grifola frondosa*, *Schizophyllum commune*, and *Coriolus versicolor*. *L. edodes* also had the most potent macrophage stimulating activity in a liquid culture rather than in a solid culture. In order to improve rice bran utilization and the yield of exo-biopolymer with macrophage stimulating activity, the treatment of Rapidase effectively increased the macrophage stimulating activity (about 30% increase), whereas the other enzymes (Econase, Viscozyme, Ultraflo, Celluclast, and Thermylase) treatments did not increase the macrophage stimulating activity. Exo-biopolymer with macrophage stimulating activity from *L. edodes* contained mainly neutral sugars (58.7%) with considerable amounts of uronic acid (32.2%) and a small amount of proteins (9.1%). Component sugars of exo-biopolymer consisted of mainly arabinose, galactose, glucose, mannose, and xylose (0.95:0.81:0.96:1.00: 0.39, respectively). When the exo-biopolymer was treated with $NaIO_4$, $NaClO_2$, and pronase, the $NaClO_2$ treatment and pronase digestion had little effect, whereas $NaIO_4$ oxidation significantly decreased the macrophage stimulating activity (47.6% reduction at 100 μg/ml). Therefore, the carbohydrate moiety in exo-biopolymer from *L. edodes* plays an important role in the expression of the macrophage stimulating activity.

Key words: Exo-biopolymer, *Lentinus edodes*, macrophage stimulating activity, submerged culture

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Mushrooms have long been attracting a great deal of interest in many areas of foods and biopharmaceutical industries, and are regarded as popular or effective medicines for treatment of various human diseases, such as hepatitis, hypertension, hypercholesterolemia, and gastric cancer [13, 34]. In particular, much interest has been generated in the subject of exo-polysaccharides produced by fungi, due to their various biological and antitumor activities [12]. Many biologically active polysaccharides have been isolated from various fungi [16, 30] and some of these polysaccharides, such as lentinan from *Lentinus edodes* fruit bodies [23], schizophyllan from *Schizophyllum commune* culture filtrates [28], grifolan from *Grifola frondosa* [26], and PSK from *Coriolus versicolor* culture mycelia [31] are now used in clinics. These polysaccharides enhance and stimulate the immune system of humans and mice [9, 29], and are thus called biological response modifiers. Moreover, the polysaccharides stimulate the immune functions of T lymphocytes and the nonspecific immune functions mediated by natural killer (NK) cells and macrophages [7].

The strain employed was a newly screened strain of *L. edodes*, producing exo-biopolymers from rice bran in submerged culture. The importance of *L. edodes*, the second most popular edible mushroom in the global market, is attributed not only to its nutritional value, but also to possible medical and food industrial applications. Lentinan [a polysaccharide with a backbone of β-(1→3)-D-glucan and side chains of both β-(1→3)-D- and β-(1→6)-linked D-glucose residues] isolated from the fruiting body acts as a host defense potentiator. Augmentations of NK, cytotoxic T lymphocytes (CTL), lymphokine-activated killer (LAK) cell activities, and delayed-type hypersensitivity (DTH) responses against tumor antigen were observed after

administration of lentinan [27]. Although the mechanism of antitumor activity of lentinan is not clear, lentinan is thought to augment the immune response via modulation of the function of phagocytes such as macrophages [25]. Besides its antitumor activity, it has also been demonstrated to increase the host resistance to bacterial and viral infections [11].

In the present work, the macrophage activities of exo-biopolymers produced from submerged mycelial cultures of higher fungi were tested for screening purposes. Characterization and production of exo-biopolymers with macrophage stimulating activity by *L. edodes* were studied to find new usage of rice bran, which has been used for animal feed and oil extraction.

MATERIALS AND METHODS

Microorganism Cultivation and Preparation of Exo-Biopolymer

Nine types of higher fungi (*Grifola frondosa, Lentinus edodes, Cordyceps militaris, Cordyceps sinensis, Agricus blazei, Flammulina velutipes, Auricularia auricular-judae, Schizophyllum commune*, and *Coriolus versicolor*) were isolated from Kyonggi-do in Korea. The medium used for a liquid culture contained the followings (l^{-1}): rice bran 20 g, $NaNO_3$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, KH_2PO_4 2.5 g, and pH was adjusted to 6.0. The liquid culture was incubated in 300-ml Erlenmeyer flasks containing 50 ml medium with a rotary shaker (120 rpm, 5 days) at 25°C. After centrifugation (3,000 ×*g* for 20 min), the supernatant was mixed with 4 volumes of absolute ethanol, stirred vigorously and kept overnight at 5°C. The precipitated exo-biopolymer was centrifuged at 3,000 ×*g* for 20 min, the supernatant was discarded, and exo-biopolymer was then lyophilized to be used for the assay of macrophage stimulating activity.

A solid culture was conducted in 500-ml Erlenmeyer flask containing 80 g of rice bran and 100 ml of distilled water. After sterilization by autoclaving, the flask was inoculated with a 10% inoculum level and incubated at 25°C for 15 days. The seed culture was grown in a 250-ml flask containing 50 ml of liquid medium at 30°C on a rotary shaker incubator (120 rpm for 5 days). After solid culture for 15 days, 250 ml of distilled water were added to the culture mass. The mixture was homogenized by Ultra-turrax T-50 (IKA Laboratories, Staufen, Germany; 7,000 rpm, 20 min), and decocted at 100°C to a half volume (2 times). After the extracts were combined and filtered through Toyo filter paper No. 2 (Toyo Roshi, Tokyo, Japan) to remove the insoluble materials, the filtrate was mixed with 4 volumes of absolute ethanol, stirred vigorously, and kept overnight at 5°C. Exo-biopolymer was obtained by the same method as for liquid culture.

Chemical Assay of Exo-Biopolymer

Total carbohydrate, uronic acid, and protein were determined by the phenol-H_2SO_4 [4], *m*-hydroxydiphenyl [1], and Bradford's method [2] with Bio-Rad dye (Bio-Rad Laboratories, Hercules, CA, U.S.A.), respectively, using galactose, galacturonic acid, and bovine serum albumin as the respective standards. Component sugars of the polysaccharides were converted to the corresponding alditol acetates after hydrolysis with 2 M TFA for 1.5 h at 121°C [10], and analyzed by GLC (gas-liquid chromatography) according to the procedure of Zhao *et al.* [36]. GLC was carried out on a Hewlett-Packard 6890 II gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with an SP-2380 capillary column (0.2 μm film, 0.25 mm i.d.× 30 m, Supelco, Bellefonte, PA, U.S.A.). Temperature program was: 60°C for 1 min, 60→220°C (increasing 30°C/min), 220°C for 8 min, 220→250°C (increasing 8°C/min), and held at 250°C for 15 min. The molar ratios were calculated from the peak areas and response factors using the flame-ionization detector.

Chemical and Enzymatic Treatments of Exo-Biopolymer

Periodate oxidation and pronase digestion of exo-biopolymer were performed according to the method of Yamada *et al.* [33]. For $NaIO_4$ oxidation, macrophage stimulating exo-biopolymer (50 mg) was stirred in 50 mM acetate buffer (pH 4.5) containing 25 mM $NaIO_4$ (50 ml) at 4°C for 4 days in the dark. The oxidized products were reduced with $NaBH_4$ and dialyzed to obtain the periodate-oxidized exo-biopolymer. In addition, exo-biopolymer from *L. edodes* (20 mg) was digested with Actinase E (20 mg, Kaken Pharmaceutical Co., Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 7.9) containing 10 mM $CaCl_2$ (20 ml) at 37°C for 2 days. After the reaction was terminated by neutralization with 0.1 N HCl, the mixture was dialyzed and lyophilized to obtain the pronase-digested exo-biopolymer.

Chlorite treatment was carried out by the method of Oka *et al.* [17]. Exo-biopolymer (20 mg) was dissolved in 4% acetic acid (20 ml), and $NaClO_2$ (100 mg) was added. The solution was stirred at 70°C until the color changed and was neutralized with 3 N NaOH in an ice bath. The reaction products were dialyzed and lyophilized to obtain the chlorite-treated exo-biopolymer.

Exo-Biopolymer Production by Enzyme Addition in a Liquid Culture

After the medium (50 ml) for a liquid culture, including rice bran (1 g), was autoclaved at 121°C for 15 min, enzyme solution (5 mg/ml) was added to the medium. The liquid culture was incubated in 300-ml Erlenmeyer flasks containing 50 ml medium with a rotary shaker (120 rpm, 5 days) at 25°C. After incubation for 5 days, the culture broth was heated in a boiling water bath for 10 min to stop the

Table 1. Characteristics of various enzyme preparations.

Enzyme	Source	Characteristics
Econase	*Trichoderma* sp.	Cellulase preparation
Rapidase	*Aspergillus niger* & *Trichoderma longibrachiatum*	Multienzyme complex including pectinase, hemicellulase, and cellulase
Viscozyme	*Aspergillus* sp.	Multienzyme complex including arabinase, cellulase, β-glucanase, hemicellulase, and xylanase
Ultraflo	*Humicola insoles*	Multiactive β-glucanase including cellulose, xylanase, pentosanase, and arabinase
Celluclast	*Trichoderma reesei*	Cellulase preparation
Amylase	*Aspergillus oryzae*	Amylase preparation

enzyme reaction. After centrifugation (3,000 ×*g* for 20 min), the supernatants were mixed with 4 volumes of absolute ethanol, stirred vigorously, and kept overnight at 5°C. The precipitated exo-biopolymer was centrifuged at 3,000 ×*g* for 20 min, discarding the supernatant. Exo-biopolymer was lyophilized to be used for the assay of macrophage stimulating activity. Characterization of enzymes for the degradation of rice bran is shown in Table 1.

Macrophage Stimulating Activity

Six- to eight-week old ICR male mice (Daihan-Biolink Co., Korea) were injected interperitoneally with 1 ml of 3% thioglycolate medium. After 3 days, macrophage cells were prepared from the peritoneal cavity of mice by washing twice with 5 ml of cold RPMI-1640 medium containing 5 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml). An aliquot (200 μl) of the cell suspension (1×10^6 cells/ml) was seeded in a flat-bottomed 96-well microplate. After incubation for 2 h at 37°C in a humidified atmosphere of 5% CO_2-95% air, non-adherent cells were removed by washing twice with RPMI-1640 medium. The adherent macrophage monolayer was used for the following experiments [8]. Macrophage stimulating activity was measured by the procedure of Suzuki *et al.* [25] with slight modification. The adherent macrophage cells were cultured in the presence of test samples in a 96-well microplate for 24 h. Macrophage monolayer in a 96-well microplate (1×10^5 cells/ml) was solubilized by the addition of 25 μl of 0.1% Triton X-100. One-hundred-fifty microliter of 10.0 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added to the reaction mixture, and absorbance at 405 nm was photometrically measured, using a microplate reader (Bio-Rad Laboratories, Model 3550-UV).

Statistical Analysis

All results are expressed as mean±S.E. The difference between the control and sample in these experiments was tested for statistical significance by student's *t*-test. A value of $p<0.05$ was considered to indicate statistical significance.

Results and Discussion

Macrophage Stimulating Activities of Various Mushroom Mycelia

Nine different higher fungi (*G. frondosa*, *L. edodes*, *C. militaris*, *C. sinensis*, *A. blazei*, *F. velutipes*, *A. auricular-judae*, *S. commune*, and *C. versicolor*) were cultured in the rice bran liquid medium for 5 days to select a suitable strain for the exo-biopolymer with macrophage stimulating activity. Among the exo-biopolymers produced from submerged cultures of mycelia tested, *L. edodes* showed the highest activity, followed by *G. frondosa*, *S. commune*, and *C. versicolor* (Fig. 1). The exo-biopolymer is composed of a tightly packed network of both arabinoxylans and cellulose. During fermentation of the rice bran, arabinoxylans and cellulose are simultaneously degraded [32]. The exo-biopolymer is produced from the degraded rice bran during the submerged culture.

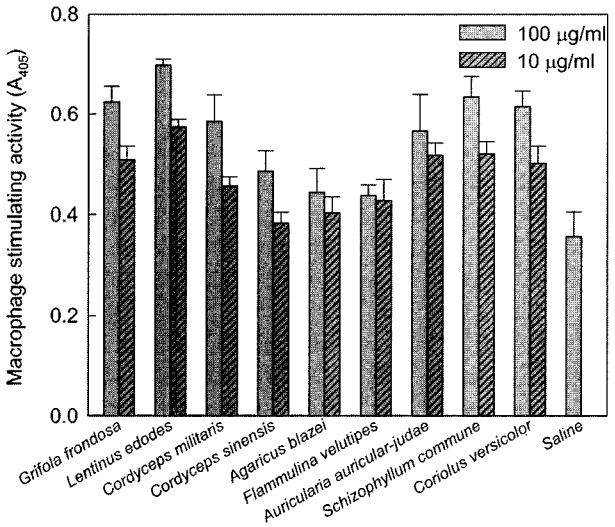

Fig. 1. Macrophage stimulating activities of exo-biopolymers from submerged cultures of nine fungi with rice bran.
Macrophage stimulating activity is expressed as the stimulation of cellular lysosomal enzymes, compared with that of the saline control, and values represent mean±S.E. of quadruplicate cultures.

There are several biopolymers produced from mushrooms such as lentinan from *L. edodes*, schizophyllan from *S. commune*, and PSK from *C. versicolor*, which are presently commercially available as antitumor agents. Administration of these compounds has been shown to inhibit the growth of various transplantable tumors in experimental animals and increases the survival rate. These compounds are considered to exert their antitumor activities through potentiation of the host animal's defense system rather than through direct inhibition of tumor cell growth [14].

Immune responses are influenced by exposure to several types of foreign substances. In the immune response, macrophages play critical roles, since they act as a link between the innate and acquired immune system, fight infection, inflammation, and angiogenesis, and promot wound healing [24]. Macrophages kill microorganisms, tumeor cells, and damaged tissues during inflammation by two separate oxidative pathways, involving the synthesis of superoxide anion (O_2^-) and nitric oxide (NO) by NADPH oxidase and nitric oxide synthases (iNOS), respectively [15]. These cells are also able to produce a variety of cytokines, such as interleukin (IL), interferon (IFN), tumor necrosis factor (TNF), and active substances like prostaglandin [19]. These results suggest that exo-biopolymers produced from submerged culture of *L. edodes* with rice bran would be important in the killing of tumor/microorganisms in innate and adaptive immune response and could represent an immunopotentiator and biological response modifiers.

Macrophage Stimulating Activity in Liquid and Solid Cultures of *L. edodes*

In order to find a new utilization for rice bran, which has been used for animal feeds and oil extraction, the above mentioned strains were examined for whether they could produce exo-biopolymer with macrophage stimulating activity from rice bran. As shown in Fig. 2, *L. edodes* had the most potent macrophage stimulating activity in a liquid culture rather than in a solid culture. The yield of exo-biopolymer in a liquid culture (16.2 mg/ml) was higher than that in a solid culture (13.4 mg/ml). This result suggested a possibility that macrophage stimulating activity was increased with the increase of exo-biopolymer production during the cultivation (data not shown). Therefore, macrophage stimulating activity appeared to closely relate with the increase of exo-biopolymer.

Therefore, a liquid culture was selected to produce exo-biopolymer with macrophage stimulating activity, because of short cultivation time and potent macrophage stimulating activity. Many investigators have attempted to cultivate mushrooms on solid artificial media rather than submerged culture [18]. Submerged culture gives rise to potential advantages of higher mycelial production in a compact space and shorter cultivation time with lesser changes of contamination [5]. In addition, exo-biopolymer with macrophage stimulating activity can concurrently be produced.

Although many workers have attempted to obtain exo-biopolymer with immune enhancing activity by a liquid culture process, very little information is available regarding the nutritional factors affecting mycelial growth and exo-biopolymer production in liquid culture. To elucidate the requirements of large-scale exo-biopolymer production from *L. edodes*, further studies are ongoing.

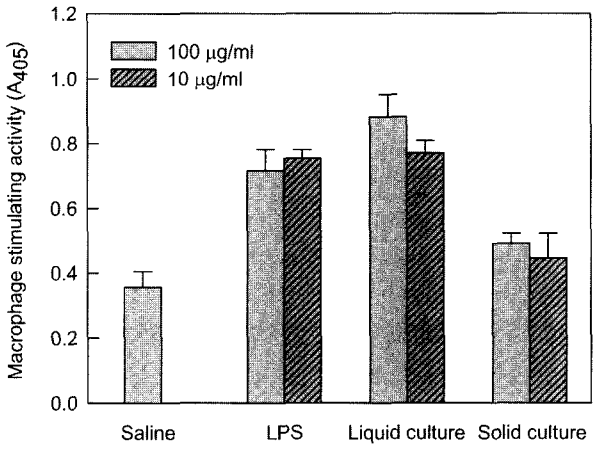

Fig. 2. Comparison of macrophage stimulating activity of exo-biopolymer between liquid-culture and solid-culture with rice bran.
Macrophage stimulating activity is expressed as the stimulation of cellular lysosomal enzymes compared with that of the saline control, and values represent mean±S.E. of quadruplicate cultures. LPS (lipopolysaccharide), the positive control.

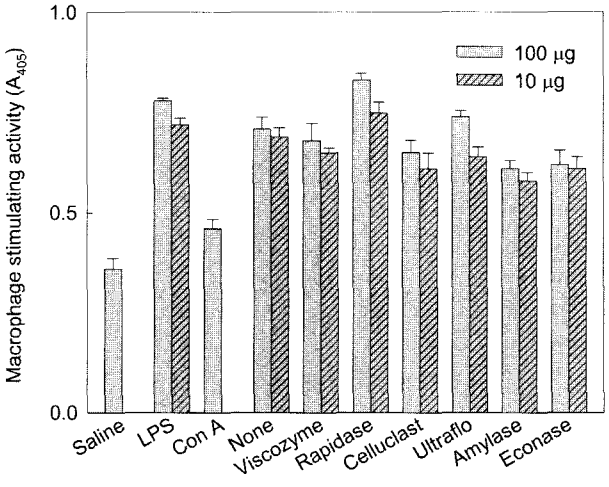

Fig. 3. Macrophage stimulating activities of exo-biopolymers produced by various enzyme preparations in submerged culture of *L. edodes* with rice bran.
Macrophage stimulating activity is expressed as the stimulation of cellular lysosomal enzymes compared with that of the saline control, and values represent mean±S.E. of quadruplicate cultures. LPS (lipopolysaccharide), the positive control.

Macrophage Stimulating Activity of Exo-Biopolymer by Enzymes in a Liquid Culture

Since the rice bran is composed of cellulose, hemicellulose, and pectic substances [22], the addition of cellulose-, xylan-, and pectin-degrading enzymes to the medium with rice bran is expected to improve material utilization and yield of exo-biopolymer with macrophage stimulating activity. As shown in Fig. 3, the addition of Rapidase (multi-enzyme complex including pectinase, hemicellulase, and cellulase) effectively increased the macrophage stimulating activity (about 30% increase), whereas the other enzymes did not increase the macrophage stimulating activity. Yoshizawa *et al.* [35] also reported that the addition of commercially available cellulose- and hemicellulose-degrading enzymes improved the digestion of steamed rice, and Fukuda *et al.* [6] has also shown that the addition of a crude enzyme solution, which exhibits cellulose-, xylan-, and pectin-degrading activities, improved material utilization and alcohol yield in sake mash. This is probably due to the structure of the rice endosperm cell wall, which is very tightly associated with the cellulose and hemicellulose (arabinoxylan) network [3]. Because of heterogeneity in the composition and structure of rice endosperm cell wall, a multienzyme can be effective for the biodegradation of these polysaccharides, and the addition of Rapidase may be effective for degrading the side chains from the hairy regions of the structure.

Characterization of Exo-Biopolymer from a Submerged Culture of *L. edodes* with Rice Bran

Macrophage stimulating exo-biopolymer from a submerged culture of *L. edodes* with rice bran, a crude polysaccharide fraction, contained mainly neutral sugars (58.7%) with considerable amounts of uronic acid (32.2%) and a small amount of proteins (9.1%) (Table 2). Component sugar analysis showed that the exo-biopolymer consisted of mainly arabinose, galactose, glucose, mannose, and xylose (molar ratio of 0.95:0.81:0.96:1.00:0.39). In addition, when

Table 2. Physicochemical properties of macrophage stimulating exo-biopolymer from submerged culture of *L. edodes* with rich bran.

Chemical composition	Content (%)
Neutral sugar	58.7
Uronic acid	32.2
Protein	9.1
Component sugar	**(mol. %)**
Rhamnose	4.0
Fucose	1.7
Arabinose	21.8
Xylose	9.0
Mannose	22.9
Glucose	22.0
Galactose	18.6

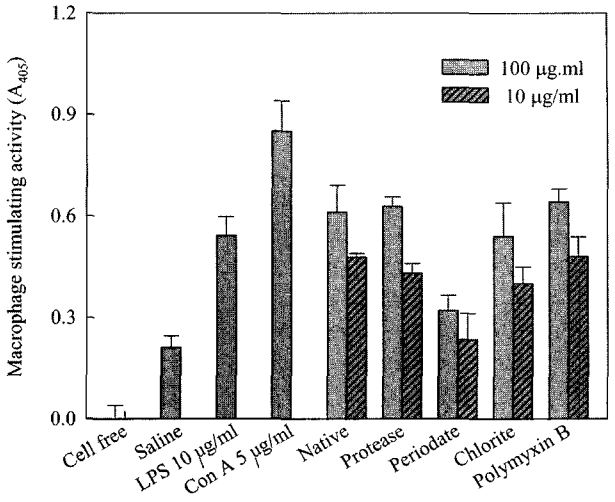

Fig. 4. Effect of chemical and enzymatic treatment of exo-biopolymer from submerged culture of *L. edodes* with rice bran on macrophage stimulating activity.
Macrophage stimulating activity is expressed as the stimulation of cellular lysosomal enzymes compared with that of the saline control, and values represent mean±S.E. of quadruplicate cultures. LPS (lipopolysaccharide), the positive control.

the macromolecular fraction, macrophage stimulating exo-biopolymer, was treated with $NaIO_4$, $NaClO_2$, and pronase in order to find out which moiety in exo-biopolymer contributed to the expression of the activity, $NaClO_2$ treatment and pronase digestion had little effect, whereas $NaIO_4$ oxidation of exo-biopolymer significantly decreased macrophage stimulating activity (47.6% reduction at 100 μg/ml) (Fig. 4). Therefore, it was suggested that the carbohydrate moiety in the *L. edodes* exo-biopolymer plays an important role in the expression of the macrophage stimulating activity. Compositional differences were evident between lentinan of *L. edodes* fruiting bodies and exo-biopolymer from a submerged culture of *L. edodes* with rice bran. A polysaccharide, having a backbone of β-(1→3)-D-glucan and side chains of both β-(1→3)-D- and β-(1→6)-linked D-glucose residues, was the major component of lentinan [20], whereas the exo-biopolymer consisted of mainly carbohydrates with various type of sugars, such as arabinose, galactose, glucose, mannose, xylose, and uronic acids at the active site. Arabinose is present mainly in the arabinoxylan fraction of rice bran. Sugar composition of the monocotyledon (i.e., rice, wheat, and maize) cell walls (rich in arabinose, xylose, and glucose) is characteristic for the monocotyledon primary cell wall, which is composed of mainly arabinoxylans and cellulose [32]. However, the rice cell wall is mainly composed of glucose, which is mostly probably present as cellulose and some xyloglucan [21]. Exo-biopolymer composition varies, depending on the structure of the monocotyledon cell wall, which is a very tightly associated cellulose and hemicellulose (arabinoxylan) network. Due to this tight association,

arabinoxylans and glucose are degraded at the same rates, and one cannot be degraded without degradation of the other. These results suggest that exo-biopolymer is produced from the degradation of rice bran during the submerged culture.

The fact that macrophage stimulating activity of the exo-biopolymer described in the present study decreased significantly by periodate oxidation (Fig. 4) suggests an important role of the carbohydrate moiety in the expression of the exo-biopolymer activity. Exo-biopolymer is rich in neutral sugars, such as arabinose, galactose, glucose, and mannose, and uronic acid (Table 2). It is reasonable to assume that the exo-biopolymer from *L. edodes* contained mainly hemicelluloses, such as arabinoxylan and glucomannan, and pectic polysaccharides. However, further studies on the purification and characterization of the structure of the exo-biopolymer are needed.

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