동충하초 균사체 최적 성장을 위한 심부배양 조건에 따른 형태학적 변화 및 균사체 열수 추출물의 면역학적 특성

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Morphological Changes by Submerged Culture Conditions for the Mycelial Optimal Growth of *Cordyceps sinensis* and Immunological Properties of Hot Water Extract of Mycelium

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Abstract The mycelial dispersed growth of *Cordyceps sinensis* was optimized in submerged batch culture at initial pH of 5.0, 150 rpm, and 25℃. The morphological data showed much more dispersed growth of *C. sinenesis* at initial pH of 5.0. Also, projected area, main hyphal length and number of tips for the mycelial growth of initial pH 5.0 were higher than those of other initial pHs. The industrial medium for mycelial production of *C. sinensis* was determined to be molasses of 100 g and crushed brewery yeast of 10 g per liter as carbon and nitrogen sources, respectively. With these culture conditions, the maximum production of mycelia was approximately 30.0 g per liter by batch culture in 5-liter jar fermenter with no controlled pH. This result suggests that large-scale mycelia production of *C. sinensis* may be possible in submerged batch culture. The hot water extract of mycelia from *C. sinensis* was mainly composed of 83.0% carbohydrate, 11.8% protein, 1.9% lipid, and 2.4% ash and there were present glucose, mannose, galactose, and arabinose as molar ratio of 8.79 : 2.59 : 1.34 : 1.0 in the carbohydrate, respectively. In the experiment using spleen cell and macrophage, the extract showed potent mitogenic and immuno-stimulating activities and among various components, an important factor that contribute to the immunological activities was turned out to be carbohydrate moiety.

Keywords: *Cordyceps sinensis*, immune modulating activity, morphological change, mycelial growth, submerged culture

INTRODUCTION

Cordyceps is a single genus of insect parasites in the order Clavicitaceae, belonging to the division of Ascomycota [1,2]. Insects infected by *Cordyceps* are mainly distributed in the orders of Cleoptera, Diptera, Hemiptera, Hymemoptera, and Lepidoptera in nature [3]. *Cordyceps* infects commonly insect larvae or mature insects with spores before the cocoon is formed and the fruiting bodies of the *Cordyceps* are formed and grow in the dead host. Some species have been identified as producing bioactive materials against insects [4]. In addition to *Cordyceps sinensis, C. hawkesii, C. martialis*, *C. militaris*, *C. ophioglossoides*, and *C. soborifera* have also been used in traditional oriental medicine [5].

Cordyceps sinensis has long been known to improve

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immune system, to prevent disease occurrence, and to increase strength of living body. It was reported that chemical components, pharmacological actions and clinical applications of *C. sinensis* and their cultured hypae have been made as an integrative statement in China [6]. It showed that cultured hypae from *C. sinensis* also affected many links of the immune system such as mononuclear phagocyte system.

In our previous studies, the mycelial extracts from *C. sinensis* have been reported to activate immune system, anti-stress, anti-fatigue, and some physiological activities. The hot water extract from mycelia of cultured *C. sinensis* showed activation of macrophages, proliferation of bone marrow cell mediated by Peyer's patch cells and the production of cytokines in an *ex vivo* experiment [7], and also showed the potential to reduce the level of low density lipoprotein (which is quantitatively the most significant) [8]. We investigated the chemical components, the effects of the hot water fraction, and the most potent fraction in enhancing immune activity against stimulus-induced fatigue and stress *in vivo* using rats and mice [9].

As cultivation for the production of fruiting bodies has not been developed successfully and the mycelial fermentation products have been demonstrated to have the same or stronger pharmacological efficacy than the wild *C. sinensis* [10,11], the production of mycelia by submerged fermentation is viewed as a promising alternative for natural products.

Growth in submerged cultures has the potential advantage of high mycelial mass production in specified conditions. Although nutritional requirements of some species of Cordyceps (Fr.) Link grown in submerged culture have been determined [12-15], such growth has not previously been demonstrated in *C. sinensis*. Also, to quantify the compounds used in cultivation media and to improve the mycelial mass production of *C. sinensis*, a synthetic or semi-synthetic medium is required.

In this study, we investigate economical medium and culture conditions on mycelium growth and production of *C. sinensis* in submerged batch culture, and some immune modulating activities and chemical properties of the mycelium were examined.

MATERIALS AND METHODS

Seed Culture

C. sinensis grown on potato dextrose agar was mixed with distilled water and their spores were suspended by the method as described by Song *et al.* [16]. Two percent of the suspended spores solution was inoculated into 250 mL Erlenmeyer flask containing 100 mL of potato dextrose broth and was incubated on rotary shaker at 25℃, 150 rpm for 3 days. And the culture broth with mycelia and spores was homogenized aseptically in Sorvall omni-mixer for 3 min in an ice bath. Mycelial suspended solution with spores of 1×10^7 per milliliter was used as seed culture to investigate further study.

Batch Culture

To investigate pH, carbon and nitrogen sources, and inoculum size of seed culture for optimizing culture condition, the following factors were studied periodically : 1) Temperature: The inoculated flasks were incubated at 20, 25, 28, 30℃. 2) pH: The medium was adjusted to different pHs with 1 N HCl or 1 N NaOH. The final pHs were adjusted 4.0 to 9.0 with interval of 0.5 before sterilization. 3) Seed age: One to four days old seeds are inoculated to set optimal seed age. 4) Inoculum size : To set proper inoculum volume of seed culture, two to thirty percentage (v/v) were applied. 5) Carbon and nitrogen source : cellobiose, fructose, glucose, lactose, maltose, sucrose and molasses are used as carbon source. And crushed brewery yeast powder, soybean meal, yeast extract, ammonium sulfate, ammonium nitrate, and corn steep liquor were tested as nitrogen source.

To prepare nitrogen sources suitable for optimal culture conditions, the sources were sterilized separately from other components containing sugars if necessarily. The pH was adjusted with 1 N NaOH or 1 N HCl prior to sterilization.

The batch flask culture was carried out with 250 mL Erlenmeyer flask containing 100 mL of culture broth. The batch fermentation in the stirred-jar fermenter (5 L, KF Co., Korea) was carried out with working volume of 3 L and air flow rate of 1.0 vvm.

Morphological Observation and Image Analysis

Cultured mycelia were observed under electron microscope (Axiolab microscope; ZEISS, Germany). Also the effect of culture condition on morphological changes of mycelia was carefully examined during the submerged culture. Briefly, the cell morphology was studied on photomicrographs with optical microscopy connected with Image Pro 3.0 software. A digital camera was mounted on a microscope and the video signal of the 30-fold diluted culture sample was sent to a computer capable of image processing and analysis. Once an image was captured in digital form, it could be processed to improve its quality or to select interesting features, and then analyzed to obtain the information of morphological factors. Morphological factors such as hyphal length, number of tips, number of spores and length of swollen hyphal fragments were measured after sorting and classifying by Image analyzing process.

Each image was analyzed automatically with Image Pro 3.0 software.

Dry Cell Weight

After centrifuging ten milliliter of culture broth at 12,000 × g for 10 min and filtering through a pre-weighed filter (Whatman GF/C Cat No. 1822 047), the obtained filter was washed twice with distilled water and dried at 95℃ to measure dry weight of mycelia.

Preparation of Hot Water Extract from Mycelium of *C. sinensis*

The dried mycelia of *C. sinensis* were decocted in water, followed filtration with metal mesh, and the residues were re-extracted by the same procedure (3 times). The extracts were centrifuged to remove insoluble materials, and lyophilized to obtain hot-water extract (21.7% yield).

Periodate Oxidation

The procedure was performed as described previously [17]. Briefly, hot-water extract from the dried mycelia of *C. sinensis* (50 mg) was dissolved in 50 mM acetate buffer (pH 4.5, 40 mL), and stirred in 50 mM acetate buffer containing 100 mM $NaIO_4$ at 4℃ for 96 h in the dark. The oxidized products were reduced with $NaBH_4$ and dialyzed to obtain the products (63.3% yield).

Mitogenic Activity

Primary cultures of spleen cell were used to measure the mitogenic activity. Splenocytes were obtained by passing pieces of spleen through a stainless mesh, treated with a hypotonic solution to lyse erythrocytes, and washed 3 times with PBS. The viability of the splenocytes was more than 95%, as assessed by the trypan blue dye exclusion method. Whole splenocytes were suspended in RPMI-1640 medium supplemented with 10% FCS and then used for experiments. Cells were washed and incubated at a density of 2×10^5 cells/mL in RPMI-1640 medium with the samples at 37℃ for 3 days in a humidified atmosphere of 5% CO_2-95% air. The mitogenic activity was measured by MTT assay with slight modification of the procedure of Sugawara *et al.* [18]. Six hour prior to culture termination, 20 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL in PBS) was added to each cell and the cells were continuously incubated. On terminating the culture incubation, the medium was removed. Then, DMSO was added to dissolve formazan crystals formed in cells, and the absorbance was measured at 490 nm by microplate reader (Bio-Rad, Model 3550-UV, Hercules, CA, USA).

Macrophage stimulating Activity

Male 6- to 8-week old ICR 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 the 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 (1×10^6cells/mL) was seeded in a flat-bottomed 96-well microplate. After incubation for 2 h at 37℃ 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. Macrophage stimulating activity was measured by the procedure of Suzuki *et al.* [19] 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) were 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) was added to the reaction mixture, and the absorbance at 405 nm was photometrically measured using a microplate reader (Bio-Rad, Model 3550-UV, Hercules, CA, USA).

Analysis of Sugar Components

Total carbohydrate and protein content were determined by the phenol-sulfuric acid [20] and Lowry method [21] using glucose and bovine serum albumin as standards. Briefly, carbohydrate composition of the polysaccharide was analyzed as alditol acetates after hydrolyzing the polysaccharide with 2 M trifluoroacetic acid (TFA) for 1.5 h at 121℃ by gas liquid chromatography (GLC) using an SP-2380 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco, Bellefonte, PA, USA) and equipped with a flame ionization detector (Young-Lin Co., Ltd., Seoul, Korea). Dry oxygen-free helium (flow rate, 1.5 mL/min) was used as the carrier gas. The temperature profile used was programmed at 60℃ for 1 min, increased by each 30℃ per min to 215℃, held at 215℃ for 18.8 min, followed by an increase of each 8℃ per min to 250℃, and then held at 250℃ for 5.7 min. Molar ratios were calculated from peak areas and molecular weights.

Data and Statistical Analysis

Experimental results recorded were means ± standard deviation (SD) of triple determinations. The data were

analysed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Duncan's multiple range tests at $p < 0.05$ or independent sample T test ($p < 0.05$). Results were processed by SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Morphological Changes and Image Analysis

To optimize the growth of *C. sinensis*, it is necessary that morphological factors should be analyzed quantitatively. Accordingly, morphological factors were measured quantitatively on morphological change by image analysis using digital camera and Image Pro program, (Table 1 and 2). *C. sinensis* was grown in the range of initial pH 4.0-9.0 for 5 days in batch flask culture containing potato dextrose broth. In culturing at initial pH of 4.0, final pH was changed to pH 6.6, and hyphal growth was examined to be weakly dispersed as can be seen in Table 1. When initial pHs were adjusted between 5.0 and 7.0, final pHs were changed to pH 7.1-7.2. At initial pH of 5.0, the morphological data showed much more dispersed growth of *C. sinenesis*. In case of initial pHs of 8.0 and 9.0, final pHs were varied to approximately pH 7.3, and the mycelia of *C. sinensis* grew as pellet type. Also, projected area, main hyphal length and number of tips for the mycelial growth of initial pH 5.0 were higher than those of other initial pHs. Accordingly, these results indicated that initial pH of 5.0 is suitable for optimal mycelia growth of *C. sinensis* than other initial pHs. Pirt and Callow [22] investigated the influence of pH on growth of *Penicillium chrysogeum*. That is, filamentous growth occurred at pH 6.0, while at a higher pH up to pH 7.4, pellet formation took place together with shorter and thicker hyphae. Whitaker and Long [23] have reported that *Aspergillus niger* grew in a filamentous form at pH 5.0. At pH 6.0, 7.0 and 8.0, pellets of various types were formed depending on the spore concentration in the inoculum. Bae *et al.* [24] reported that submerged growth of *Paecilomyces japonica* varies from the pellet to the filamentous form depending on the growth medium, physical environment, aeration and agitation, etc. However, among all factors, culture pH was the most important parameter to significantly affect morphological change. Also, the filamentous form of *Paecilomyces japonica*was observed in cultivating at pH 5.0 and 6.0, but pellet formation in cultivating at pH of both 4.0 and 7.0-9.0.

Next, *C. sinensis* was grown according to the culture time (2-5 days) at initial pH of 5.0 in batch flask culture containing potato dextrose broth. At two day of culture, a few filamentous hyphae and some swollen hyphal fragments were observed in culture broth. As shown in Table 2, mycelia growths were maintained with dispersed hyphal type as culture time increased. At 5 days of incubation, there were examined many filamentous hyphae, swollen hyphal fragments, and tips. Matsumura *et al.* [25] reported that short swollen hyphal fragments are rapidly differentiated into spores. Also, a large number of studies on the effect of culture conditions on fungal morphology have been reported [26,27]. Main hyphal length of *C. sinensis* was changed approximately from 22 to 118 μm and maximum diameter of the mycelia get more thick from 1.3 to 4.2 μm, and its number of tips was from 2 to 15 for incubation period of 5 days (Table 2). This data indicated that projected area, hyphal length and mycelia tips of *C. sinensis* were rapidly increased at the end of incubation time (5 days).

Table 1. Effect of initial pH on morphological factors for mycelia growth of *C. sinensis* in submerged culture

pH	Area (μm^2)[a]	Main hyphal length (μm)[b]	Major axis[c]	Number of tips[d]
4.0	2410	98.01	2.86	7
5.0	2736	117.58	4.19	15
6.0	2674	84.30	4.35	17
7.0	2570	57.34	3.62	14
8.0	2472	47.30	2.47	11
9.0	2418	46.23	1.40	6

[a] Projected area of the freely dispersed or clumped mycelia.
[b] The longest connected path through the cell.
[c] Maximum mycelial diameter.
[d] The number of branches (growing tips).

Table 2. Effect of culture time on morphological factors for mycelial growth of *C. sinensis* in submerged culture

Days	Area (μm^2)[a]	Main hyphal length (μm)[b]	Major axis[c]	Number of tips[d]
2	2484	21.98	1.30	2
3	2503	86.31	3.05	7
4	2677	98.75	3.76	14
5	2736	117.58	4.20	15

[a] Projected area of the freely dispersed or clumped mycelia.
[b] The longest connected path through the cell.
[c] Maximum mycelia diameter.
[d] The number of branches (growing tips).

Effect of Nitrogen and Carbon Source

Nitrogen sources were usually required for enhancement of mycelial growth and morphological differentiation. It has been studied for the optimization of nitrogen source [28]. Organic nitrogen sources gave higher mycelial growth compared to inorganic source [24,29]. Two percent of seed culture was inoculated into broth medium containing carbon and nitrogen sources. The submerged culture was

carried out at 25℃, 150 rpm and initial pH 5.0.

Crushed brewery yeast powder, soybean meal, yeast extract, and corn steep liquor, ammonium sulfate, and ammonium nitrate were tested as nitrogen source and combination of nitrogen sources (0.05%, w/v) and carbon sources (2%, w/v) was utilized for mycelial growth. As a result, crushed brewery yeast powder and yeast extract proved to be good nitrogen sources when using xylose, maltose, glucose, cellubiose, fructose, sucrose, lactose, and arabinose as carbon source (Fig. 1). The crushed brewery yeast powder was better nitrogen source in using maltose and sucrose as carbon source, while yeast extract was best nitrogen source in using glucose as carbon source. Organic nitrogen sources gave rise to higher mycelial growth compared to inorganic sources. This result is very similar to the observations for fungal fermentation [24,29]. Though molasses contains unpurified sucrose and glucose, but its unknown elements could play an important role in proliferating mycelial growth of another fungus, *Lentinus edodes* [30]. Corn steep liquor (CSL), widely used for chief nitrogen source, was added into the culture medium to improve the productivity [31]. However, in our experiment, 0.05% CSL didn't make a role to increase the mycelial growth, as shown in Fig. 1.

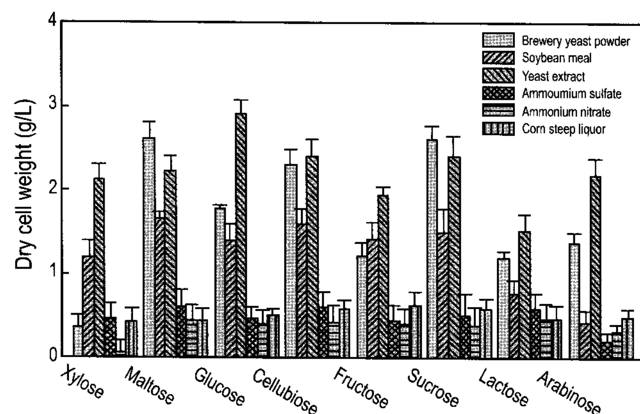

Fig. 1. Effect of carbon and nitrogen sources on mycelial growth of *C. sinensis* in submerged culture. Culture was carried out at 25℃, 150 rpm, and initial pH 5.0 for 5 days in PDB medium with various carbon sources and inoculated with seed of 2% (v/v), carbon sources (2%), nitrogen sources (0.05%).

As another factor to affect on the mycelial growth of *C. sinensis*, it was investigated that the maximum growth of mycelia was dependent on the inoculum size when using glucose and sucrose as carbon source and yeast extract or crushed brewery yeast as nitrogen source as can be seen in Fig. 2 and Fig. 3. Inoculum size of twenty percent gave better yield than those of other inoculum sizes. That is, as inoculums size increases, the growth yield proportionally was enhanced.

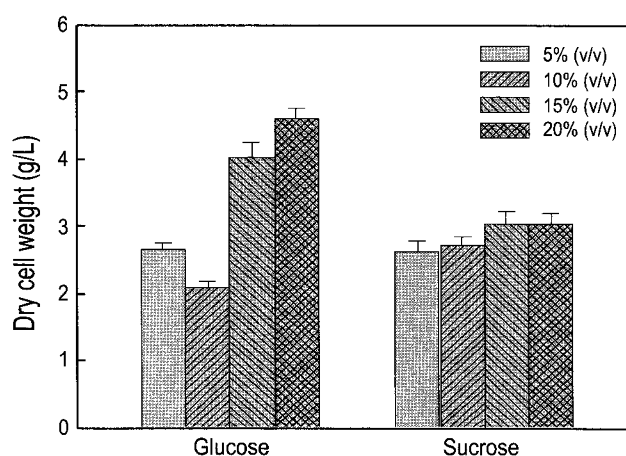

Fig. 2. Effect of inoculum size and carbon sources on mycelial growth *C. sinensis* in submerged culture, using yeast extract as a nitrogen source. Culture was carried out at 25℃, 150 rpm, and initial pH 5.0 for 5 days in PDB medium with various carbon sources: final concentrations ofcarbon source (2%) and nitrogen source (0.05%).

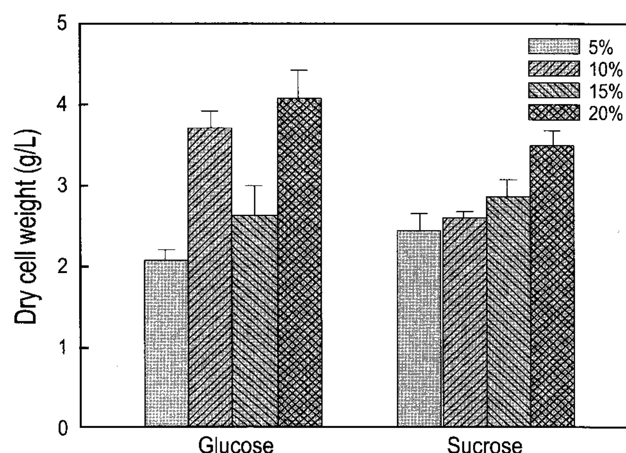

Fig. 3. Effect of inoculum size and carbon sources on mycelial growth of *C. sinensis* in submerged culture, using crushed brewery yeast powder as a nitrogen Source. Culture was carried out at 25℃, 150 rpm, and initial pH 5.0 for 7 days in PDB medium with various carbon sources final concentrations of carbon source (2%) and nitrogen source (0.05%).

To investigate mycelial productivity of *C. sinensis* using a 5 L Jar fermenter in industrial medium, we used the medium consisting of molasses (100 g/L) and crushed brewery yeast powder (0.5 g/L), and incubated the culture with seed culture of 20% (v/v) at initial pH of 5.0, 150 rpm and 25℃ on basis of preliminary tests. As shown in Fig. 4, mycelial growth was constantly increased during 5 days of the culture, while the reducing sugar was proportionally decreased and exhausted at the end of culture. Finally, maximum mycelia growth (dry cell weight of 30 g/L) was achieved at the end of incubation using a 5-L Jar fermenter. This result suggests that large-scale mycelia

production of *C. sinensis* may be possible in submerged batch culture.

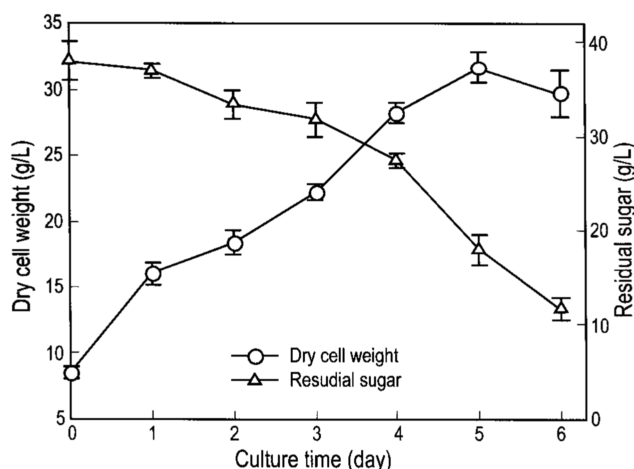

Fig. 4. Profileof mycelial growth of *C. sinensis* in submerged culture of 5-L jar fermenter. Culture was carried out at 25℃, 150 rpm, and initial pH 5.0 for 6 days in the medium containing molasses of 10%, crushed brewery yeast 0.5%, with seed culture of 20% (v/v).

Chemical Properties of Hot Water Extract

The hot water extract of mycelia from *C. sinensis* was mainly composed of 83.0% carbohydrate, 11.8% protein, 1.9% lipid, and 2.4% ash and there were present glucose, mannose, galactose, and arabinose as molar ratio of 8.79 : 2.59 : 1.34 : 1.0 in the carbohydrate, respectively (Table 3). This result suggested that the hot water extract of *C. sinensis* consisted of glucomannan with minor sugars.

Table 3. The chemical composition and component sugar of hot water fraction of mycelium from cultured *Cordyceps sinensis*.

Component	Content (%)[c]
Carbohydrate	83.9 ± 0.2
Protein[a]	11.8 ± 0.1
Lipid	1.9 ± 0.1
Ash	2.4 ± 0.1
Component sugar[b]	**Relative Content (mol. ratio)**
Glucose	1
Mannose	0.8
Galactose	0.5
Arabinose	0.1

[a] To determine crude protein, the 6.25 conversion factor was used.
[b] Component sugar of polysaccharide was determined by GC as alditol acetate derivatives and analyzed by GLC using an SP-2380 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco, U.S.A.) equipped with an FID.
[c] All values expressed are mean of triplicate.

Also, polysaccharide showing anti-oxidatiive activity was isolated from *C. sinensis* containing glucose, mannose and galactose in a ratio of 1.0:6.0:0.75 [32]. The difference in sugar composition or molar ratio was supposed to due to the composition of culture medium and the sugar moiety may affect on different biological activities.

Immunological Activity of Hot Water Extract from the Mycelium of *C. sinensis*

Macrophages play a critical role in all phases of host defense which are both innate and adaptive immune response. Infectious organism must first adhere to the epithelial cells and then cross epithelium. A local non-adaptive immune response helps repel the infection and delivers antigen to local lymph nodes, leading to adaptive immunity and clearance of the infection. The relative macrophage stimulating activity of hot water extract from the mycelium of *C. sinensis* increased to 1.8-fold than negative control (physiological saline) (Fig. 5).

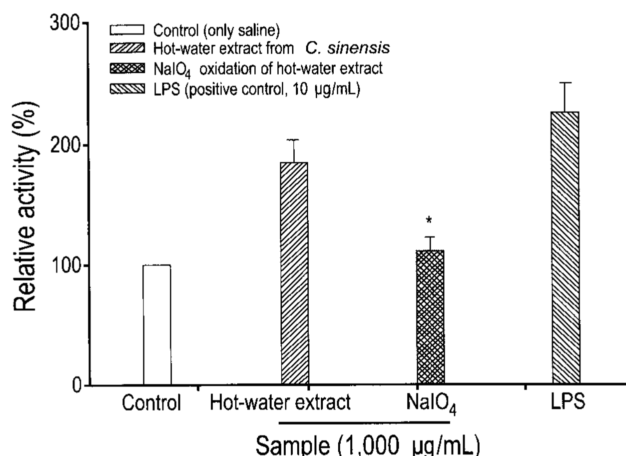

Fig. 5. Effect of $NaIO_4$ oxidation for hot-water extract of mycelium from *C. sinensis* on macrophage activity.

This is similar to the effect of *Agaricus bispours* fruiting bodies extract on the production of cellular lysosomal enzyme in mouse macrophage [33]. Also, hot water extract from the mycelium of *C. sinensis* had a potent mitogenic acivity compared to negative control (Fig. 6). In order to elucidate which moiety contributed to the immunological activities, when the extract was treated with $NaIO_4$ and oxidized, both activities of the oxidized extract were decreased by 40% of the macrophage stimulation activity and 27.9% of the mitogenic activity, respectively, compared to those of the hot water extract (Fig. 5 and 6). Accordingly, this result indicated that the carbohydrate moiety of hot-water extract might be related with the activities. In conclusion, it is important that the dispersed mycelial growth and morphological changes by the optimal culture

conditions of *C. sinensis* may increase the macrophage stimulating and mitogenic activities of exo- and endo-polysaccharides.

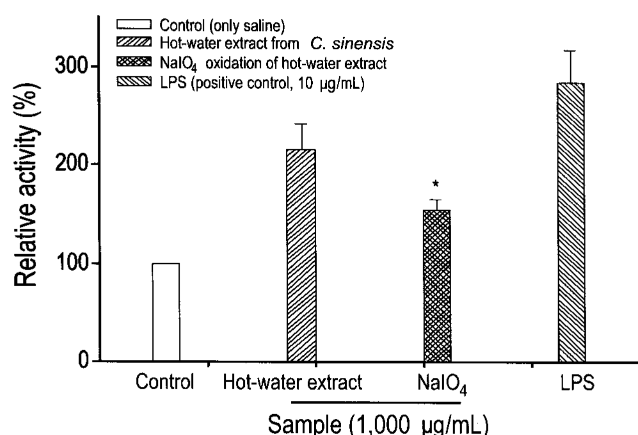

Fig. 6. Effect of $NaIO_4$ oxidation for hot-water extract of mycelium from *C. sinensis* on mitogenic activity.

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