Food Sci. Biotechnol. Vol. 17, No. 1, pp. 95 ~ 101 (2008)

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Biological Activities and Partial Characterization of *Beauveria bassiana* Mycelium

Sung-Yong Park[1], Hyuk-Hwan Song, Yong-Gab Lee[1], Cheol-Sik Yoon[2], and Chan Lee*

Department of Food Science and Technology, Chung-Ang University, Ansung, Gyeonggi 456-756, Korea

[1]Ottogi Research Center, Anyang, Gyeonggi 431-070, Korea

[2]Research Institute of Engineering and Technology, Korea University, Seoul 136-701, Korea

Abstract Some biological activities of *Beauveria bassiana* were studied to elucidate pharmacological function of *B. bassiana*-infected larva of the silkworm. The mycelium consisted mainly of carbohydrate (65.8%), followed by protein (15.9%) and fat (8.3%). Glucose (68.8%), mannose (7.1%), and galactose (6.1%) were major components in carbohydrates. Ten amino acids including glutamine, threonine, valine, aspartic acid, alanine, leucine, serine, glycine, arginine, and isoleucine were found in protein as major amino acids. Various extracts were prepared from the freeze-dried mycelium of *B. bassiana* by systemic extraction and their biological activities were investigated. Among tested fractions, the hot-water extract (HW) contributed significantly to the anti-coagulant activity, anti-complementary activity, and stimulation of intestinal immune system. The methanol extract (ME) increased acetylcholinesterase (AChE) inhibition activity and reactive oxygen species (ROS) scavenging activity.

Keywords: *Beauveria bassiana*, anti-coagulant, anti-complement, instestinal immunity, acetylcholinesterase

Introduction

The genus *Cordyceps* (Ascomycotina, Pyrenomycetes, Clavicipitales, Clavicipitaceae) is known as a group of entomophatogenic fungi and is used in traditional oriental medicines in northeastern Asia. It was reported in a previous study that this genus is composed of approximately 750 species, and that some species produce toxins against insects (1). The interest of researchers in medicinal plants and mushrooms as natural sources of active principles has increased noticeably over the past several decades, and particular attention has been paid to components of various traditional Asian medicines. *C. militaris*, *C. sinensis*, *C. ophioglossoides*, and *Beauveria bassiana* have potent immunological activities. *C. militaris* in particular is a herbal ingredient that has been used in oriental medicine on patients suffering from cancer, and it has also been used at the East-West Cancer Center of Daejeon University's Oriental Hospital (Daejeon, Korea) since 2001 (2). *C. militaris* also inhibited proliferation of cultured human glomerular mesangial cells induced by low-density lipoprotein (3), and it exhibited anti-inflammatory (4) and hypocholesterolemic effects (5). Some of the immuno-modulatory and antitumor activities of *C. sinesis*, *C. ophioglossodies*, and *C. scarabaecola* have previously been reported (6-9).

The genus *Beauveria*, which is known as an imperfect state of *Cordyceps*, also has potential in herbal medicines as well as biocontrol agents against insect pests. Traditionally, *B. bassiana*-infected larvae of the silkworm has been used as a medicine to treat strokes in Korea (10). In our previous study, it was reported that extract from *B. bassiana* synnemata has various pharmacological activities, including antioxidant and immunostimulatory functions (11). There is, however, limited knowledge of the spatial distribution and population dynamics of the species, especially above ground, which is essential to understanding the pharmaceutical activities of mycelium extract. As well, little is known about its immunoregulatory effects.

Recently, we developed an artificial medium for the mass production of mycelium of *B. bassiana*, thus providing an opportunity for further pharmacological examination. In this study, we determined whether solvent extract [ethylacetate (EA), methanol (ME), and hot-water (HW)] from *B. bassiana* mycelia has various pharmacological activities including anti-coagulant, anti-complementary, anti-AChE, antioxidant, and mucosal-immunity stimulatory function. The HW showed the most potent pharmacological activities.

Materials and Methods

Microorganisms and materials *B. bassiana* strain, collected from Seoraksan(Mt.), South Korea, was provided by Dr. C.S. Yoon (Korea University, Korea) (11). *B. bassiana* was cultured in potato dextrose broth (Gibco, Grand Island, NY, USA) for 7 days (12). Mycelium of *B. bassiana* produced from the medium were freeze-dried and used for further experiments. RPMI-1640 medium and Hank's balanced salt solution (HBSS) were obtained from Gibco. Heat-inactivated fetal bovine serum (FBS) was obtained from Cell Culture Laboratories (Cleveland, OH, USA), and penicillin, streptomycin, and amphotericin B were purchased from Flow Laboratories (Irvine, Scotland, UK). Alamar Blue™ was a product of Alamar Bio-Sciences Inc. (Sacramento, CA, USA). Acetylthiocholine iodide (ASCh) and 5,5'-dithiobis-(2-nitro)-benzoic acid (DTNB) were purchased from Sigma Co. (St. Louis, MO, USA). The activated partial thromboplastin time (aPTT) reagent was obtained from Boehring Inc. (New York, NY, USA). 12-O-

*Corresponding author: Tel: +82-31-670-3035; Fax: +82-31-676-8865
E-mail: chanlee@cau.ac.kr
Received June 28, 2007; accepted August 23, 2007

Tetradecanoylphorbol-13-acetate (TPA) was a product of Research Biochemicals International (Natick, MA, USA). All other reagents were analytical grade.

Cells and culture conditions PC12 cells originated from rat pheochromocytoma and human promyelocytic leukemia HL-60 cells obtained from the Health Science Research Resources Bank. The cells were cultured in an RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco). The cells were maintained at 37°C in an atmosphere of 95% air/5% CO_2 incubator and a subculture was created by mechanically removing them from the substratum with squirts of fresh medium.

Preparation of various fractions by systematic extraction Freeze-dried mycelium were blanched at 100°C for 5 min and homogenized with Ultra-turrax T-50 (7,000 rpm, 20 min; IKA-Labortechniker, Staufen, Germany). After centrifugation (8,000×g, 30 min), the supernatant was removed and the precipitant was initially fractionated with ethylacetate, which is a solvent with a low polarity, followed by fractionation with methanol and water in increasing order of their polarity (Fig. 1).

Chemical analysis Moisture, crude protein, crude carbohydrate, crude fat, and crude ash in the mycelium were determined according to the methods of AOAC methods (13). To determine the crude protein, a 6.25 conversion factor was used. All values expressed are the means of triplicate determinations.

Analysis of component sugar For analysis of component sugar of the polysaccharide, the polysaccharide was hydrolyzed in 2 M trifluoroacetic acid at 100°C for 4 hr for galactose, glucose, and mannose analysis (14). The sugars were separated and quantitated on CarboPac PA1 column (2.5×250 mm, 4.5×250 mm, Dionex, Sunnyvale, CA, USA) with a Bio-LC DX-600 (Dionex) using a CarboPac PA1 cartridge (2.5×50 mm, 4.5×50 mm). Five-tenth M NaOH and distilled water were used as an eluant at a flow rate 0.25 or 1 mL/min. Duplicated data was analyzed using PeakNet on-line software.

Amino acid analysis For determination of the amino acid composition, sample was hydrolyzed and PITC labeled by the Pico-Tag method (Waters, Milford, MA, USA). Phenylisothiocyanate-derivatized free amino acids were applied to a Waters Pico-Tag column (3.9×300 mm) equilibrated with buffer A, equipped with a Waters HPLC system (510 HPLC pump, gradient controller, 717 automatic sampler, 996 photodiode array detector) and eluted with a linear gradient composed of buffer B (0, 14, 20, 46, and 100%) at a flow rate of 1 mL/min. Absorbance was measured at 254 nm. Buffer A was 140 mM sodium acetate (6% acetonitrile), and buffer B was 60% acetonitrile. Analytical method development, data collection, and data integration were performed using Millennium^{32} Chromato-graphy manager software (15,16). The sample was analyzed in triplicate.

Anti-coagulant and anti-complementary activity The aPTT was measured at 37°C with an automatic blood

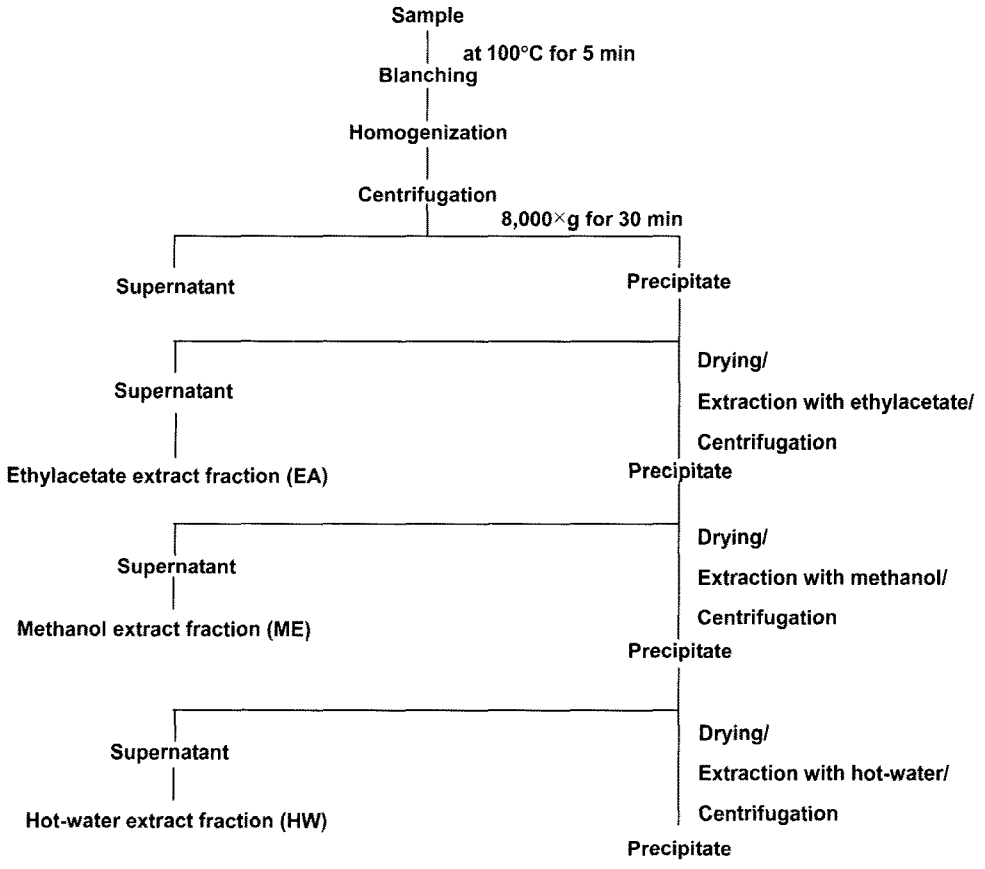

Fig. 1. Preparation of various fractions by systematic extraction.

coagulator (Clot-1A; Hospitex Diagnostics, Milan, Italy) according to the method described in a previous study (17). Briefly, a sample of 100 μL of human citrated platelet-poor plasma (1 : 10 v/v, 3.8% sodium citrate) was warmed at 37°C, and then 100 μL of a pre-warmed activator reagent was added. The mixture was incubated at 37°C for 18 sec. Pre-warmed 20 mM calcium chloride (100 μL) was then added, and aPTT was recorded as the time for clot formation (18).

Anti-complementary activity was measured according to the procedure described by Yamada *et al.* (19). Normal human serum (NHS) was obtained from a healthy adult. Various concentrations of the sample in water (50 μL) were mixed with 50 μL of NHS and 50 μL of gelatin veronal-buffered saline (GVB^{++}, pH 7.4) containing 500 μg of Mg^{++} and 150 μg of Ca^{++}. The mixtures were pre-incubated at 37°C for 30 min, and 350 μL of GVB^{++} was added. IgM-hemolysin-sensitized sheep erythrocytes (250 μL) at 1×10^8 cells/mL were added to the mixtures diluted serially (10-160 folds), and then incubated at 37°C for 1 hr. After addition of phosphate-buffered saline (PBS, pH 7.2) and centrifugation, the absorbance of the supernatants was detected at 412 nm. The NHS was incubated with water and GVB^{++} as a control. Anti-complementary activity was expressed as the percentage inhibition of the total complementary hemolysis (TCH_{50}) of the control (20). The degree of inhibition of TCH_{50} was calculated thus:

Inhibition (%)
$=100\times[(TCH_{50}$ of control$-TCH_{50}$ of sample$)/TCH_{50}$ of control]

Intestinal immune system modulating activity The activity was measured according to the procedure of Hong *et al.* (21). A 180 μL of Peyer's patch cell suspension (2×10^6 cells/mL in RPMI 1640-FBS) prepared from the small intestine of C3H/HeN mice (5 to 7 weeks old, Daehan Biolink Co. Korea) was cultured with 20 μL of the test sample in a 96-well flat-bottom microtiter plate for 5 days at 37°C in a humidified atmosphere of 5% CO_2-95% air (22). The result of culture supernatant (50 μL) was incubated with bone marrow cell suspension (2.5×10^5 cells/mL) from C3H/HeN mice for 6 days. After 20 μL of Alamar Blue™ solution was added to each well, the cells were then continuously cultured for 5-24 hr. The fluorescence intensity was measured to determine cell numbers with Spectrafluor Plus (Tecan, Austria) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm during cultivation. The intestinal immune system modulating activity was expressed as the stimulation of cell growth of the bone marrow cells compared with that of the control in which Peyer's patch cells were incubated with distilled water instead of the test sample.

AChE inhibition activity The AChE Inhibition assay was performed according to the method of Ellman *et al.* (23,24) using ASCh as a substrate. To obtain an enzyme source, PC12 cell cultures were homogenized in a Glass-Col homogenizer with 5 volumes of buffer (10 mM Tris-HCl, pH 7.2 containing 1 M NaCl, 50 mM $MgCl_2$, and 1% Triton X-100), and centrifuged at 10,000×g for 30 min. The result of supernatant was used as an enzyme source. The rates of hydrolysis by AChE were monitored spectrophotomatically using a 96-well microtiter plate format. Each freeze-dried extract (10 μL) dissolved in 5% DMSO was mixed with enzyme solution (10 μL), and incubated at 37°C for 15 min. Absorbance was read at 405 nm immediately after the addition of 70 μL of Ellman reaction mixture [0.5 mM acetylthiocholine and 1 mM DTNB in 50 mM sodium phosphate buffer (pH 8.0)] to the mixture of enzyme and extract. Readings were repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. A blank reaction was measured by substituting saline for the enzyme. The percentage of enzyme activity value in the inhibitors was calculated by comparison with those of the control's activity (100%). The degree of enzyme inhibition was calculated thus:

Inhibition (%)
$=100\times(1-$inhibited reaction/uninhibited reaction$)$

Inhibition activity of TPA-induced O_2^- generation An inhibitory test of TPA-induced O_2^- generation in DMSO-differentiated HL-60 cells was performed as previously reported (25). In order to determine the inhibitory effect of O_2^- generation, a test compound dissolved in 5 μL of DMSO was added to a DMSO-induced differentiated HL-60 cell suspension in PBS (1 mL). After incubation at 37°C for 15 min, the cells were washed twice with PBS to remove unabsorbed test compound. TPA (10 nM), and cytochrome C solution (1 μg/mL) were added to the reaction mixture which was then incubated for another 15 min. The reaction mixture was placed on ice to terminate the reaction. After centrifugation at 250×g, a visible absorption at 550 nm was measured. Inhibitory effects were expressed by a decreasing ratio of absorbance of test compounds relative to a control experiment.

Statistical analysis Statistical significances were determined by one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc analysis, and results were expressed as mean±SD for 2 independent experiments. Differences were considered significant for $p<0.05$.

Results and Discussion

Chemical composition *of B. bassiana* mycelium In order to investigate the chemical composition, we first determined the chemical components in mycelium of *B. bassiana*. As shown in Table 1, the mycelium provides a beneficial source of crude carbohydrate (65.8%) and protein (15.9%). However, the mycelium also contained small amount of crude fat (8.3%) and ash (4.9%). The carbohydrates in the mycelium included a large quantity of glucose (86.8%), in addition to mannose (7.1%) and galactose (6.1%) (Table 2). The mycelium also contained 17 amino acids, and glutamine, threonine, valine, aspartic acid, and alanine were present in large quantities. Trace amount of other amino acids were detected at a concentration below 3 mg/g (Table 3). Compositional differences were evident between the synnemata of *B. bassiana* and its mycelium. The synnemeta exhibited less carbohydrate content than mycelium. Mannose was the major component of carbohydrate in the synnemaeta (11).

Table 1. Proximate and chemical properties of freeze-dried mycelium of *B. bassiana*

Component	Content (%)[2)]
Moisture	2.4
Crude protein[1)]	15.9
Crude fat	8.3
Crude carbohydrate	68.5
Crude ash	4.9

[1)]To determine crude protein, a 6.25 conversion factor was used.
[2)]All values expressed are means of triplicate determinations.

Table 2. Component sugar of polysaccharide of mycelium of *B. bassiana*

Component sugar[1)]	Content (%)[2)]
Glucose	86.8
Mannose	7.1
Galactose	6.1
Arabinose	Trace
Xylose	Trace

[1)]Component sugar of polysaccharide was determined by CarboPac PA1 column (2.5×250 mm, 4.5×250 mm, Dionex), with a Bio-LC DX-600 (Dionex), using a CarboPac PA1 cartridge (2.5×50 mm, 4.5×50 mm).
[2)]All values expressed are means of triplicate determinations.

Table 3. Amino acid composition of mycelium of *B. bassiana*

Component amino acid[1)]	Content (mg/g)[2)]
Alanine	3.10
Arginine	2.80
Aspartic acid	3.26
Cysteine	0.46
Glutamine	4.72
Glycine	2.88
Histidine	1.85
Isoleucine	2.51
Leucine	3.03
Lysine	0.86
Methionine	0.37
Phenylalanine	1.99
Serine	2.94
Threonine	3.93
Tryptophan	0.02
Tyrosine	0.26
Valine	3.56

[1)]The amino acid was analyzed by Pico-Tag method (Waters).
[2)]All values expressed are means of triplicate determinations.

Anti-coagulation activity of HW and ME Blood coagulation is a major cause of mortality and disability, leading to cerebral hemorrhage, myocardial infarction, peripheral ischemia, arteriosclerosis, and pulmonary embolism (26). In the last few years, there have been extensive investigations into therapeutic agents for the development of effective anti-coagulants, because of coagulation results in cardiovascular and cerebral thrombosis (26). Recently, it has been reported that it is possible to isolate anti-coagulants from oriental medicinal mushrooms, such as *Ganoderma lucidium* (27) and *Pleurotus ostreatus* (28). Because anti-coagulants have been used for lowering blood pressure and for detoxification of snake venom, the activity could be useful in clinical applications for reducing thrombosis. We thus sought to investigate whether the extracts promote anti-coagulation activity by aPTT methods. As shown in Fig. 2, HW (68.9±3.3 sec) and ME (41.2±2.7 sec) from *B. bassiana* significantly delayed clotting time, while EA (19.3±2.1 sec) did not affect clotting time at all. The control was 20.4±2.5 sec. HW and EA reached approximately 3- and 2-fold higher levels than the control value. Similar activity was found in HW fraction from synnemeta of *B. bassiana* (11).

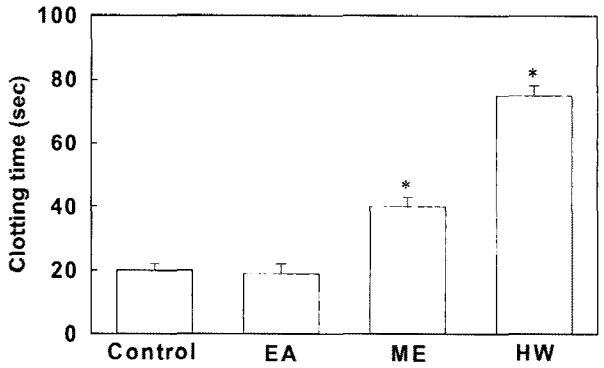

Fig. 2. Anti-coagulant activities of mycelium of *Beauveria bassiana* fractionated by solvent extraction. The final concentration of the sample was 100 μg/mL. Values represent the means± SD (n=4), *$p<0.05$. Significance between the control and samples. Control (saline); EA, ethyl acetate extract fraction; M, methanol extract fraction; HW, hot-water extract fraction.

This result may suggest that HW and ME from mycelia of *B. bassiana* contained significant anti-coagulant activity *in vitro*. These extracts may be useful in clinical applications for anti-coagulant activity.

Increases of anti-complementary activities by HW Immunostimulating anti-complementary substances have been recognized as characteristic biological response modifiers. Anti-complement activity plays an important role in host resistance in the primary humoral mediation of Ag-Ab reaction. Recently, a considerable number of medicinal herbs and plants have been found to possess immunostimulating and anti-complementary effects, and to have higher anti-tumor activities (29,30). The activity is usually reported in polysaccharides such as those from *Malva sylvestris* var. *mauritiana* (31) and *Bupleurum falcatum* (32). Therefore, anti-complementary activity in solvent extracts was tested by the complement fixation test, and the activity was presented as inhibition of TCH_{50}. As shown in Fig. 3, HW showed inhibitory effects only on the hemolytic activity of the complement system towards antibody-coated erythrocytes (63.8±4.1%) compared with the control (48±4.9%). EA and ME exhibited a decrease in inhibitory effect, 32±5.2 and 36±6.1% respectively. Similar difference in anti-complementary activity between each fraction was found in our previous report (11). From the data, HW has significant anti-complementary activity. The result shows the possibility that mycelia of *B. bassiana* may contain some kind of regulator of the complementary system.

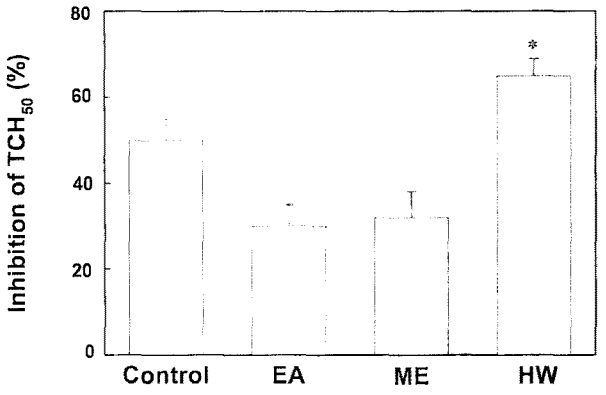

Fig. 3. Anti-complementary activities of mycelium of *Beauveria bassiana* fractionated by solvent extraction. The final concentration of the sample was 100 μg/mL. Values represent the mean± SD (n=4), *p<0.05. Significance between the positive control and samples. Control (LPS, 100 μg/mL); EA, ethyl acetate extract fraction; ME, methanol extract fraction; HW, hot-water extract fraction.

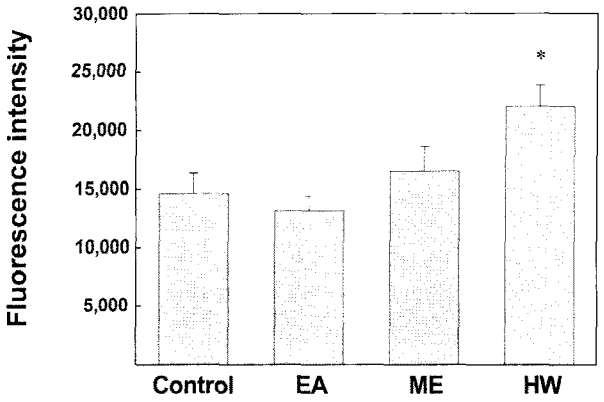

Fig. 4. Intestinal immune system modulating activities of mycelium of *Beauveria bassiana* fractionated by the solvent extraction. The final concentration of the sample was 100 μg/mL. Values represent the means±SD (n=4), *p<0.05. Significance between the positive control and samples. Control (LPS, 100 mg/mL); EA, ethyl acetate extract fraction; ME, methanol extract fraction; HW, hot-water extract fraction.

Proliferation of bone marrow cells through the intestinal immune system by HW The gut-associated lymphoreticular tissues (GALT) play an important role in host defense, including the IgA response of the intestinal immune system (33). The intestinal immune system, including Peyer's patches, not only contributes to the defense system of the mucosa, but also regulates systemic inflammation, resulting in the suppression of allergic reactions and autoimmune diseases (33). Recently, it was reported that HW of *C. sinensis* stimulates the proliferation of bone marrow cells through Peyer's path cells and macrophage activation *in vitro* and *in vivo* (34). In this study, we investigated whether the solvent extracts from submerged cultures of *B. bassiana* stimulate the proliferation of bone marrow cells through Peyer's patch cells and this effect was tested *in vitro* with fluorescence intensity. To investigate the effect of solvent extracts on how Peyer's patch cells mediated the hematopoietic responses of bone marrow cells, Peyer's patch was obtained from C3H/HeN mice, cultured with the extracts and then added to the culture of bone marrow cells. As shown in Fig. 4, HW significantly proliferated the bone marrow cells through Peyer's patch cells (1.5-fold). ME had an intermediate activity (1.2-fold) and EA showed no immune stimulation in intestinal cells. This action exhibits that HW has the most potent fraction on the proliferation of bone marrow cells mediated by Peyer's patch cells *in vitro*. It may be assumed that oral administration of HW of *B. bassiana* can induce the local stimulation in the intestinal system. In case of synnemata of *B. bassina* HW, EA, and MA exhibited similar intestinal immune system activating activity, however there was no significant differences between fractions (11).

Decrease of AChE activities by ME AChE is an enzyme, which hydrolyses acetylcholine and is used as a marker for cholinergic neural function. It is known to be involved in synaptogenesis. While it is known to be a marker for the developing chick brain, it is also implicated in neurodegenerative diseases (35,36). AChE plays an important role in Alzheimer's disease (AD), which is primarily caused by a cholinergic dysfunction. The degree of cognitive impairments in AD has been reported as correlating well with central cholinergic deficits (35). We thus sought to determine whether the solvent extracts increase the inhibitory effects of AChE activity. In order to examine the inhibition of AChE activity, the extract was treated in PC-12 cells for 24 hr and cell lysate was used as a substrate. Among the extracts tested, EA and HW exhibited low inhibition of AChE activity, 30.4±6.1 and 8.9±3.8% respectively. However, the value was lower than that of the positive control (48.6±6.4%) and ME (40.2± 4.2%) as shown in previous report related to synnemata of *B. bassiana* (11). The activity of the ME was almost the same as that of the positive control. This result indicates that ME from *B. bassiana* mycelium may be helpful in inhibiting AChE activity, but not in HW and EA.

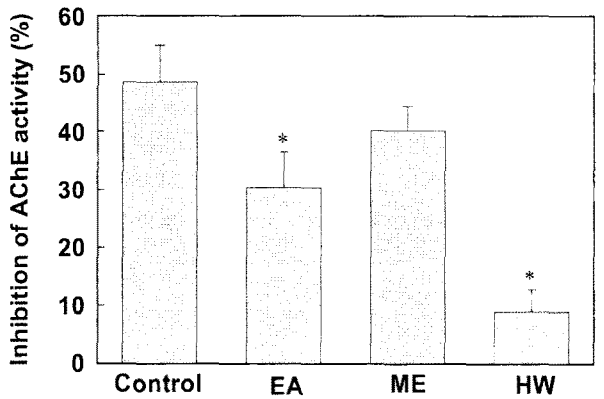

Fig. 5. Inhibitory effect on AChE activities of mycelium of *Beauveria bassiana* fractionated by solvent extraction. The final concentration of sample was 130 μg/mL. Values represent the means±SD (n=4), *p<0.05. Significance between the positive control and samples. Control (tacrine, 130 μg/mL); EA, ethyl acetate extract fraction; ME, methanol extract fraction; HW, hot-water extract fraction.

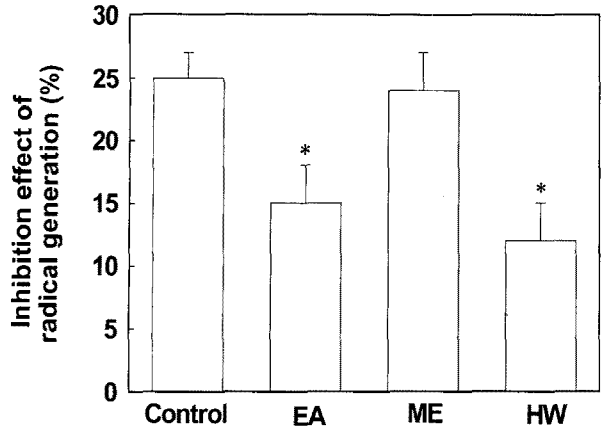

Fig. 6. Inhibitory effect on TPA-induced O_2^- generation of mycelium of *Beauveria bassiana* fractionated by solvent extraction. The final concentration of the sample was 100 μg/mL. Values represent the means±SD (n=4), *p<0.05. Significance between the positive control and samples. Control (genistine, 100 μg/mL); EA, ethyl acetate extract fraction; ME, methanol extract fraction; HW, hot-water extract fraction.

Inhibition of TPA-induced O_2^- generation by HW and EA O_2^- is one of the precursors of several types of ROS (37) resulting in oxidative stress-related diseases including cancer. Also, the production of ROS is associated with many neurodegenerative diseases (38,39). Therefore, inhibition of ROS generation is the most accepted and recognized therapeutic markers for development of cognitive enhancers. Recently, traditional medicinal plants have been used to enhance cognitive function and to alleviate other symptoms associated with AD (40,41). The present study also attempted to search for an O_2^- generation inhibitor as a promising candidate for the prevention of these diseases. Among the extracts, ME (24±4.3%) revealed the highest inhibitory activity on the TPA-induced O_2^- generation, followed by EA (14.5±3.1%) and HW (12.4±2.9%). The activity of ME was same as that of the positive control, which exhibited 24.1±3.2% activity. These extracts may be contributed to protecting against ROS-induced injury in animals. ME fraction from synnemata of *B. bassiana* exhibited much lower inhibitory activity on the TPA-induced O_2^- generation (11)

In summay, physiological activities of the freeze-dried mycelium of *B. bassiana* were evaluated. HW extract from mycelium showed biological activities including anti-complementy activity, anticogualent activity, and the most potent intestinal immune system modulating activity. The ME increased AChE inhibition activity and ROS scavenging activity. Various biological activities in the present study indicated that the mycelium of *B. bassiana* contained some healthful ingredients and that the intrinsic nature of these active compounds requires further investigation.

Acknowledgments

The research was supported by the 'GRRC' Project of Gyeonggi Provincial Government, Korea.

References

1. Sung J-M, Kim C-H, Yang K-J, Lee H-K, Kim Y-S. Studies on distribution and utilization of *Cordyceps militaris* and *Cordyceps mutans*. Korean Mycol. 21: 94-105 (1993)
2. Yoo H-S, Shin J-W, Cho J-H, Son C-G, Lee Y-W, Park S-Y, Cho C-K. Effects of *Cordyceps militaris* extract on angiogenesis and tumor growth. Acta Pharmacol. Sin. 25: 657-665 (2004)
3. Zhao-Long W, Xiao-Xia W, Wei-Ying C. Inhibitory effect of *Cordyceps sinensis* and *Cordyceps militaris* on human glomerular mesangial cell proliferation induced by native LDL. Cell Biochem. Funct. 18: 93-97 (2000)
4. Won S-Y, Park E-H. Anti-inflammatory and related pharmacological activities of cultured mycelia and fruiting bodies of *Cordyceps militaris*. J. Ethnopharmacol. 96: 555-561 (2005)
5. Koh J-H, Kim J-M, Chang U-J, Suh H-J. Hypocholesterolemic effect of hot-water extract from mycelia of *Cordyceps sinensis*. Biol. Pharm. Bull. 26: 84-87 (2003)
6. Yamaguchi Y, Kagota S, Nakamura K, Shinozuka K, Kunitomo M. Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. Phytother. Res. 14: 647-649 (2000)
7. Zhang Q, Wu J, Hu Z, Li D. Induction of HL-60 apoptosis by ethyl acetate extract of *Cordyceps sinensis* fungal mycelium. Life Sci. 75: 2911-2919 (2004)
8. Ohmori T, Tamura K, Tsuru S, Nomoto K. Antitumor activity of protein-bound polysaccharide from *Cordyceps ophioglossoides* in mice. Jpn. J. Cancer Res. 77: 1256-1263 (1986)
9. Yu K-W, Kim K-M, Suh H-J. Pharmacological activities of stromata of *Cordyceps scarabaecola*. Phytother. Res. 17: 244-249 (2003)
10. Pemberton RW. Insects and other arthropods used as drugs in Korean traditional medicine. J. Ethnopharmacol. 65: 207-216 (1999)
11. Yoon C-S, Yu K-W, Bae S-H, Song H-H, Park H-S, Lee C. Chemical properties and physiological activities of synnemata of *Beauveria bassiana*. J. Microbiol. Biotechn. 13: 125-133 (2003)
12. Fernandez S, Groden E, Vandenberg JD, Furlong MJ. The effect of mode of exposure to *Beauveria bassiana* on conidia acquisition and host mortality of Colorado potato beetle, *Leptinotarsa decemlineata*. J. Invertebr. Pathol. 77: 217-226 (2001)
13. AOAC. Official Methods of Analysis of AOAC Intl. 16th ed. Association of Official Analytical Communities, Arlington, VA, USA (1995)
14. Ha Y-W, Jeon B-T, Moon S-H, Toyoda H, Toida T, Linhardt RJ, Kim Y-S. Characterization of heparin sulfate from the unossified antler of *Cervus elaphus*. Carbohyd. Res. 340: 411-416 (2005)
15. Tarr GE, Shively JE. Methods of Protein Microcharacterization. Humana Press, Clifton, NJ, USA. pp. 155-194 (1986)
16. Rubio LA. Determination of diaminopimelic acid in rat feces by high performance liquid chromatography using the Pico-Tag method. J. Chromatogr. B 784: 125-129 (2003)
17. Fox I, Dawson A, Loynds R, Eisneer J, Findlen K, Levin E, Hanson D, Mant T, Wagner J, Maragaonre J. Anticoagulant activity of HirulogTM, a direct thrombin inhibitor, in human. Thromb. Haemostasis 69: 157-165 (1993)
18. Lee H-S. Anticoagulant properties of compounds derived from Fennel (*Foeniculum vulgare Gaertner*) fruits. Food Sci. Biotechnol. 15: 763-767 (2006)
19. Yamada H, Kiyohara H, Cyong JC, Kojima Y, Kumazawa Y, Otsuka Y. Studies on polysaccharides from *Angelica acutiloba*. Part I. Fractionation and biological properties of polysaccharides. Planta Med. 50: 163-168 (1984)
20. Kiyohara H, Yamada H, Cyong JC, Otsuka Y. Studies on polysaccharides from *Angelica acutiloba*. *V.* Molecular aggregation and anti-complementary activity of arabinogalactan from *Angelica acutiloba*. J. Pharmacobio-Dynam. 9: 339-345 (1986)
21. Hong T, Matsumoto T, Kiyohara H, Yamada H. Enhanced production of hematopoietic growth factors through T cell activation in Peyer's patches by oral administration of *kampo* (Japanese herbal) medicine, '*Juzen-Taiho-To*'. Phytomedicine 5: 353-360 (1998)
22. Jun W-J, Kim S-H, Lee D-H, Chung J-W, Sim S-I, Lee K-W, Cho H-Y, Hong B-S. *In vitro* and *in vivo* effects of *Gelidium amansii* on

intestinal immune system. Food Sci. Biotechnol. 14: 147-151 (2005)
23. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95 (1961)
24. Lim S-S, Han S-M, Kim S-Y, Bae Y-S, Kang I-J. Isolation of acetylcholinesterase inhibitors from the flowers of *Chrysanthemum indicum* Linne. Food Sci. Biotechnol. 16: 256-269 (2007)
25. Marker M, Andrews PC, Babior BM. Measurement of O_2^- production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. Method Enzymol. 105: 358-365 (1984)
26. Hirsh J, Fuster VI. Guide to anticoagulant therapy. Part I: Heparin. Circulation 89: 1449-1468 (1994)
27. Choi H-S, Sa Y-S. Fibrinolytic and antithrombotic protease from *Ganoderma lucidum*. Mycologia 92: 545-552 (2000)
28. Choi H-S, Shin H-H. Purification and characterization of a fibrinolytic protease in *Pleurotus ostreatus*. Mycologia 90: 647-679 (1998)
29. Chun H, Shin D-H, Hong B-S, Cho H-Y, Yang H-C. Purification and biological activity of acidic polysaccharide from leaves of *Thymus vulgaris L.* Biol. Pharm. Bull. 24: 941-946 (2001)
30. Kweon M-H, Hwang H-J, Sung H-C. Isolation and characterization of anticomplementary beta-glucans from the shoots of bamboo *Phyllostachys edulis*. Planta Med. 69: 56-62 (2003)
31. Gonda R, Tomoda M, Shimizu N, Yamada H. Structure and anti-complementary activity of an acidic polysaccharide from the leaves of *Malva sylvestris* var. *mauritiana*. Carbohyd. Res. 198: 323-329 (1990)
32. Yamada H, Ra KS, Kiyohara H, Cyong JC, Otsuka Y. Structural characterization of an anti-complementary pectic polysaccharide from the roots of *Bupleurum falcatum L.* Carbohyd. Res. 189: 209-226 (1989)
33. Pearay O, Jiri M, Michael L, Warren S, Jerry M, John B. Handbook of Mucosal Immunology. Academic Press, London, UK. pp. 275-285 (1994)
34. Koh J-H, Yu K-W, Suh H-J, Choi Y-M, Ahn T-S. Activation of macrophages and the intestinal immune system by an orally administered decoction from cultured mycelia of *Cordyceps sinensis*. Biosci. Biotech. Bioch. 66: 407-411 (2002)
35. Coyle JT, Price DL, DeLong MR. Alzheimer's disease: A disorder of cortical cholinergic innervation. Science 219: 1184-1190 (1983)
36. Chemnitius JM, Haselmeyer KH, Gonska BD, Kreuzer H, Zech R. Indirect parasympathomimetic activity of metoclopramide: Reversible inhibition of cholinesterase from human central nervous system and blood. Pharmacol. Res. 34: 65-72 (1996)
37. Ohigashi H, Osawa T, Terao J, Watanabe S, Yoshikawa T. Food Factors for Cancer Prevention. Springer, Tokyo, Japan. pp. 188-193 (1997)
38. Halliwell B. Reactive oxygen species and the central nervous system. J. Neurochem. 59: 1609-1623 (1992)
39. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. P. Natl. Acad. Sci. USA 91: 10771-10778 (1994)
40. Howes MR, Houghton PJ. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol. Biochem. Be. 75: 513-527 (2003)
41. Eldeen IMS, Elgorashi EE, van Staden J. Antibacterial, anti-inflammatory, anti-cholinesterase, and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. J. Ethnopharmacol. 102: 457-464 (2005)