

Anti-inflammatory Activity of the Galactomannans Produced by a New Fungal Strain *Trichoderma erinaceum* DG-312

Ji-Hoon Joo¹, Jong-Min Lim¹, Kwang-Bon Koo, Jong-Won Yun¹ and Jang Won Choi*

Department of Bioindustry, College of Life & Environment, Daegu University, Kyungsan, Kyungbuk 712-714, Korea

¹Department of Biotechnology, College of Engineering, Daegu University, Kyungsan, Kyungbuk 712-714, Korea

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The crude galactomannans (GMs) were obtained from the culture of a newly isolated fungus *Trichoderma erinaceum* DG-312 and their anti-inflammatory activity was investigated in mice. The maximum concentrations of mycelial biomass and GMs reached 9.44 g/l, 2.72 g/l at day 3 in a 5-l stirred-tank bioreactor, respectively. The results of Sepharose CL-6B gel chromatography and compositional analysis revealed that the crude GMs contain heterogeneous polysaccharides consisting of 74.9% mannose and 24.1% galactose. The GMs was shown to possess a significant anti-inflammatory activity against acetic acid-induced inflammatory mouse model in a dose-dependent manner, when mice were treated with 100 and 200 mg GMs/kg body weight. The inhibition in vascular permeability (60.6%) and in writhing response (62.5%) evidenced an anti-inflammatory activity of the GMs. The marked anti-inflammatory and writhing-lowering properties of the GMs suggest its potential therapeutic use.

Key words – Anti-inflammatory activity, capillary permeability, galactomannans, submerged culture, *Trichoderma erinaceum*

Acute inflammation is an initial response to tissue injury mediated by the release of autacoids and usually precedes the development of the immune response [19,36]. An inflammatory response is also a complex response of tissue to injury involving a network of signals that leads to activation of endothelial cells and leukocyte recruitment [1,34]. It is well established that these inflammatory diseases are currently treated with either steroidal or non steroidal anti-inflammatory drugs (NSAIDs). The anti-inflammatory drugs have a variety of problems such as leukopenia, headache, and gastrointestinal disorder [6,30]. For this reason, safer compounds with less side effects are needed.

It is well known that an acute inflammation model induced by acetic acid is used to study the effects of NSAIDs on the acute phase of inflammation [8,26,27]. The intraperitoneal injection of acetic acid causes squirming and an increase in capillary permeability that is typically associated with activation of the cyclooxygenase pathway of arachidonate metabolism producing prostaglandins (PGs) [20,25,35]. The increase of the capillary permeability in acute inflammation response is also due to the release of serotonin, histamine, bradykinin, prostaglandins, and leukotrienes [9,13].

The polysaccharides originating from higher fungi have received special attention due to their potent biological and

pharmacological activities, including immuno-stimulation, anti-tumor, hypoglycemic, and anti-inflammatory activity [4,5]. In addition, it has been reported that a variety of medicinal plants and mushrooms have potent anti-inflammatory activity as NSAIDs [10,14,15,18,23,28,29,38].

During searching mushroom-pathogenic microorganisms, we isolated a new fungus from the gills of edible mushroom *Sarcodon aspratus* (Berk) S. Ito and identified as *Trichoderma* sp. In the cultivation of mushroom, *Trichoderma* species have been widely known as major competitive or weed moulds and occasionally also as pathogens [21,22]. In addition, many *Trichoderma* strains have been identified as having potential applications in biological control and in the production of valuable biomaterials such as antibiotics and industrial enzymes [3,12,31]. However, no scientific reports are available in literature describing *Trichoderma erinaceum*.

The objective of the present study was to provide a possible anti-inflammatory effect of crude GMs of *T. erinaceum* in acute inflammation mice model with a reference drug, aminopyrine. To the best of our knowledge, this study is the first evaluation of heterogeneous polysaccharides, galactomannans for anti-inflammatory effect.

Materials and Methods

Isolation and identification of the fungus

During searching mushroom-pathogenic organisms, we

*Corresponding author

Tel : +82-53-850-6756, Fax : +82-53-850-6769

E-mail : chjawa@daegu.ac.kr

isolated a new fungus from the gills of edible mushroom *Sarcodon aspratus* (Berk) S. Ito in a mountainous restrict of Kyungbuk province, Korea. The isolated strain was phylogenetically identified by ITS-5.8S rDNA sequencing analysis. The chromosomal DNA of the strain was isolated from the fresh mycelium using Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting DNA was amplified using *Taq* polymerase (Applied Biosystem, Foster, CA, USA), with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4R [5'-CAGACTT(G/A)TA(C/T) ATGGTCCAG-3'] [7] on a Techne gene thermocycler (GMI Inc. Ramsey, Minnesota, USA) under the following conditions: 95°C-5 min, 45°C-1 min, 72°C-2 min (1 cycle); 95°C-1 min, 45°C-30 sec, 72°C-2 min (29 cycle); 72°C-10 min (1 cycle). The PCR products were separated on a 1% agarose gel and the interest DNA bands were purified using PCR clean-up system (Promega). The resulting products were cloned into the pGEM-Teasy vector (Promega) and sequenced in both directions using M13 forward and reverse primers with an automated DNA sequencer (ABI PRISM® 3700 Applied Biosystems, Foster, CA, USA). The obtained nucleotide sequence of the ribosomal DNA was compared with those of GenBank using the NCBI Blast program and sequence homology was comparatively analyzed using Clustal X program [32]. Consequently, the isolated strain was identified as *Trichoderma erinaceum* and named DG312.

Fungal Cultures

A culture of *T. erinaceum* DG-312 was maintained on a potato dextrose agar (PDA) slants stock culture stored in 25% glycerol solution at -20°C for about 2 months. Slants were incubated at 25°C for 4 days, then stored at 4°C and subcultured every 4 weeks. The fungus was initially grown on PDA medium in a petridish, and then transferred into the seed culture medium (MCM medium: 20 g/l glucose, 2 g/l meat peptone, 2 g/l yeast extract, 0.46 g/l KH₂PO₄, 1 g/l K₂HPO₄, 0.5 g/l MgSO₄ · 7H₂O) by punching out 5 mm of the agar plate culture with a self-designed cutter. Shake flask cultures were carried out in 250 ml flasks containing 50 ml of the MCM medium at 25°C for 4 days, using 4% (v/v) inocula [16]. For preparation of GMs, the fermentation medium was inoculated with 4% (v/v) of the seed culture and then cultivated for 4 days in a 5-l stirred-tank bioreactor (Ko-BioTech Co., Seoul, Korea) with a working volume of 3-l under the following conditions: controlled pH at 5.0, temperature 25°C, aeration rate 2 vvm, and agitation

speed 150 rev/min.

Preparation of the galactomannans (GMs)

Mycelium was removed from the culture broth by centrifugation (9,000 × g, 20 min). The total soluble GMs were precipitated at 4°C by adding four volumes of ethanol and then dialyzed (regenerated cellulose tubing; MWCO 12,000~14,000, Spectrum Laboratories Inc., St Broadwick, Rancho Dominguez, Canada) against tap water for three days and distilled water for two days. The ethanol precipitates of the GM components were dissolved in 0.2 M NaCl buffer to a concentration of 10 g/l, and loaded onto a Sepharose CL-6B column (2.4 cm × 100 cm; Sigma Chemical Co., St Louis, MO, USA). The column was eluted with the same buffer at a flow rate of 0.6 ml/min. The protein moiety in the GMs was monitored by measuring the absorbance at 280 nm, and the carbohydrate moiety was monitored at 480 nm. The active fractions of the GMs were pooled and lyophilized until further analysis.

Chemical analyses

The sugar composition was analyzed by a Varian STAR 3600CX gas chromatography equipped with a flame-ionization detector on a SPTM-2380 capillary column (15 m × 0.25 mm, SUPELCO Co., Bellefonte, PA, USA) with He as a carrier gas. For analysis of neutral sugars the GMs were hydrolyzed with 2 M trifluoroacetic acid (3 h at 121°C). The resulting monosaccharides were converted into their corresponding alditol acetates and identified and quantified by gas chromatography.

Animals and breeding conditions

Male ICR mice (Daehan Biolink Co., Ltd, Eumsung-Gun, Korea), weighing 18~22 g, were housed in individual stainless steel cages and acclimatized with free access to food and water for at least 1 week in an air conditioned room (23±2°C with 55±5% humidity) under a 12:12-hour light-dark cycle. The mice were fed with a commercial pellet diet (Sam Yang Co., Seoul, Korea) throughout the experimental period. The care, maintenance, and treatment of animals in these studies were performed according to the guide of laboratory animals by the NIH and IACUC of KIT/KRICT.

Experimental design

All the animals were randomly divided into five groups

with seven animals in each group: inflamed control group induced by acetic acid (IC), inflamed mice received 0.9% NaCl solution; positive control group (PC), inflamed mice treated with a positive control aminopyrine (10 mg/kg body weight); GMs-treated group (GM), inflamed mice treated with GMs at the level of 50, 100, and 200 mg/kg body weight in three groups of mice. In the preliminary experiment, the efficacy of the GMs in exhibiting anti-inflammatory potential was compared at a single dose (100 mg/kg body weight). As the GMs emerged as more potent than aminopyrine, in the main experiment, the GMs were further applied in a dose-dependent manner (50, 100, and 200 mg/kg body weight).

Nociceptive activity was tested in mice using the writhing model outlined by Miño et al[20]. In this model, we used acetic acid, stimuli known to produce inflammation as well as a writhing response in mice. The intensity of nociception was quantified by counting the cumulative number of writhes occurring between 10 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscles together with a stretching of hind limbs.

Anti-inflammation assay

Anti-inflammatory activity was evaluated by measuring acetic acid-induced vascular permeability[24,37]. Male mice (ICR, 4 weeks old, 18~20 g) in five groups were dosed orally with 50, 100 and 200 mg/kg and a positive control, aminopyrine (10 mg/kg body weight) before the intravenous injection of 1% Evans blue (10 ml/kg body weight). After injection of the dye, 1% acetic acid (10 ml/kg body weight) was injected intraperitoneally. After 20 min, the mice were sacrificed and the viscera were exposed. The abdominal cavity was repeatedly washed with 5 ml of normal saline. Then, the collected washing solution was made up of 5 ml with normal saline and centrifuged at $3000 \times g$ for 15 min. The absorbance of the supernatant was measured at 590 nm using a spectrophotometer.

Statistical analysis

The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All data were expressed as mean \pm S.E. Group means were considered to be significantly different at $P < 0.05$, as determined by the technique of protective least significant difference (LSD).

Results and Discussion

Preparation and composition of the GMs

Fig. 1 shows the typical time profiles of mycelial growth and GMs production during submerged culture of *T. erinaceum* in a 5-l stirred-tank bioreactor, where the maximum concentrations of mycelial biomass and GMs were 9.44 g/l and 2.72 g/l at 72 h, respectively. Crude water-soluble GMs were obtained from the culture filtrates using a gel filtration chromatography on Sepharose CL-6B. The compositional analysis results of the crude GMs are shown in Table 1. The constituent sugars of the crude GMs were 74.92% mannose and 24.10% galactose.

Although most physiologically important fungal polysaccharides have been identified as β -(1 \rightarrow 3) or β -(1 \rightarrow 6)-linked glucans[4,5,17,33], other heteropolysaccharides with diverse linkages have frequently been reported. For instance, the immune-enhancing polysaccharides of edible mushroom *Morchella esculenta* have been known as (1 \rightarrow 2)-linked galactomannan[11].

Anti-inflammatory activity of the GMs

In Fig. 2 and 3, GMs are obviously more effective in inhibiting writhing response to acute inflammation than capillary permeability. As clearly seen in Fig. 3, the effect of the GMs

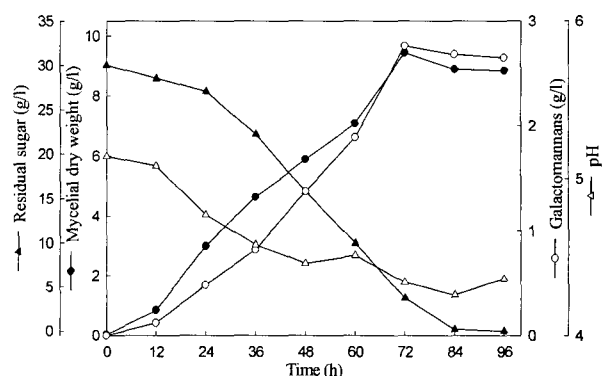


Fig. 1. Time profiles of mycelial growth and galactomannan production in a 5-l stirred-tank bioreactor.

Table 1. Compositional analysis of the polysaccharides produced by a culture of *Trichoderma erinaceum*

Sugar (%)	Composition (%)
Arabinose	Trace ^a
Xylose	Trace
Mannose	74.92
Galactose	24.10
Glucose	Trace

^aTrace means less than 1% present.

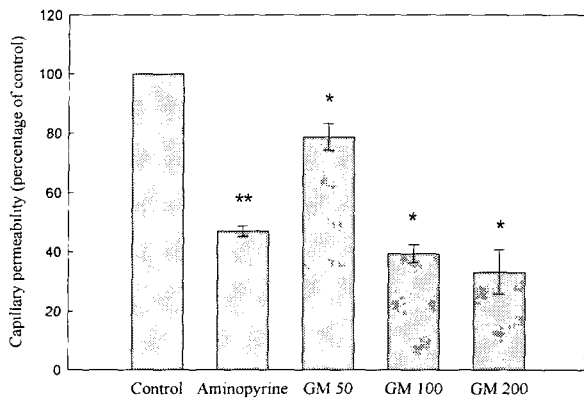


Fig. 2. Effect of *Trichoderma erinaceum* galactomannans on the vascular permeability inhibition for evaluation of anti-inflammatory activity in mice with reference to positive control aminopyrine. Doses of galactomannans (GMs) were noted in mg/kg body weight of mouse. * $P < 0.05$, ** $P < 0.01$, compared with the control group ($n=7$).

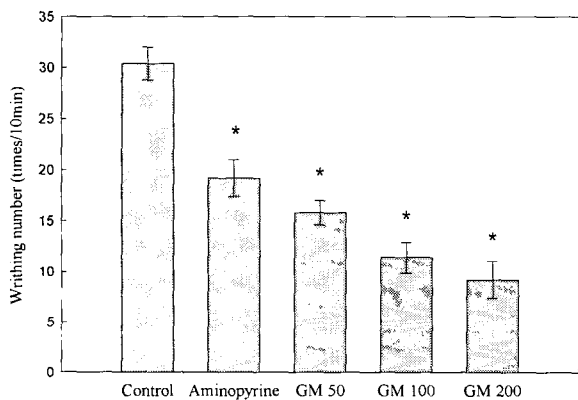


Fig. 3. Effect of *Trichoderma erinaceum* galactomannans on writhing inhibition in inflamed mice with reference to positive control aminopyrine. Doses of galactomannans (GMs) were noted in mg/kg body weight of mouse. * $P < 0.05$, compared with the control group ($n=7$).

on writhing response in mice was obvious, suggesting that GMs block capillary permeability and writhing response of acute inflammation. So, GMs may block prostaglandin and/or bradykinin release better than histamin and/or serotonin. The GMs are shown to possess a significant anti-inflammatory activity in a dose-dependent manner when treated with 50~200 mg GMs/kg body weight. The inhibition in vascular permeability (60.6%) and in writhing response (62.5%) confirmed anti-inflammatory activity of the GMs, which was higher than the standard reference drug aminopyrine at doses >100 mg/kg.

To date, several compounds with anti-inflammatory activity have been extracted from a variety of natural sources including medicinal plants[24,26,27], mushrooms[10,14,15,

18,29], and entomopathogenic fungi[38]. Diniz et al.[10] reported an inhibition of inflammatory process in rats by Tibetan mushroom, a fermented beverage composed of a dozen bacteria and yeasts living together in polysaccharide grains secreted by them. The mushroom suspensions presented an inhibition of 43% in cotton-induced granuloma and paw edema of rat model. The edema induced by carrageenin was inhibited 62%, while the dextran-induced edema was completely inhibited and antagonized the histamine edema by 52%.

Recently, Lakshmi et al.[15] have examined the anti-inflammatory activity of the mycelial extract of a medicinal mushroom *Ganoderma lucidum*, occurring in south India. This mushroom inhibited acute and chronic inflammatory edemas by 56 and 60%, respectively with a reference drug Diclofenac (10 mg/kg). It should be noted here that they used a high dose of the extract (1000 mg/kg body weight) compared to other investigations (mostly 100~200 mg/kg) [2,8,12,19]. More recently, Kim et al.[18] evaluated the plausible anti-inflammatory activity of ethanolic mycelial extract of a medicinal mushroom *Phellinus linteus*, in which dose-dependent inhibition was observed in mouse edema induced by croton oil. Among subfractions of the extracts, the *n*-butanolic fraction showed higher anti-inflammatory activity (about 40~45% with 1 mg/ear.) than other subfractions with a positive control indomethacin (71~73% with dose 0.5 mg/ear). The anti-inflammatory activity of the methanolic extract of *Pleurotus florida* Eger, an edible and commercially grown mushroom, were investigated by Jose et al.[14] in mice. The extract showed significant activity in ameliorating acute inflammation induced by carrageenan and chronic inflammation by formalin at 500 and 1000 mg/kg body weight. The effect was comparable to the standard reference drug Diclofenac. Shen et al.[29] employed a more practical evaluation method of anti-inflammatory potency for six mycelial extracts of mushroom *Antrodia camphorate* cultured under six different media against reactive oxygen species (ROS) production in peripheral human neutrophils (PMN) or mononuclear cells (MNC). Yu et al.[38] demonstrated that a widely used entomopathogenic fungus *Cordyceps militaris* possesses an anti-inflammatory activity in its extracts of the fruit body. Within the range of 50~200 mg/kg, the polysaccharide significantly suppressed ($P < 0.001$) the mouse ear edema induced by croton oil in a dose-dependent manner. At doses of 50 and 100 mg/kg, the polysaccharide also exerted a significant ($P < 0.001$) inhibitory

effect on the increased vascular permeability induced by acetic acid in mice. At a dose of 100 mg/kg body weight, the ear edema and vascular permeability were inhibited 61.5 and 55.3%, respectively.

In conclusion, like various anti-inflammatory agents with antinociceptive activity, GMs were found to possess both anti-inflammatory and antinociceptive activities. These results provide evidence that the marked anti-inflammatory and writhing reducing properties of this fungal polysaccharide suggest its potential therapeutic use in human ailments, especially vascular disorders. Future studies will focus on the exact mechanism of GMs to inflammation response. This study is the first evaluation of non-steroidal anti-inflammatory drugs (NSAID) for inflammatory activity by use of extracellular fungal fermentation product, especially galactomannans.

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초록 : *Trichoderma erinaceum* DG-312 신규 곰팡이 균주로부터 생산된 galactomannan의 항염증 활성

주지훈¹ · 임종민¹ · 구광본 · 윤종원¹ · 최장원^{*}

(대구대학교 생명환경대학 생명산업학과, ¹공과대학 생물공학과)

새로 분리된 곰팡이 *Trichoderma erinaceum* DG-312의 균사체 배양에 의해 갈락토만난(GMs)을 생산하여 쥐에서 항염증 활성을 조사하였다. 5 리터 교반 발효조에서 3일 동안 배양에 의해 얻어진 균사체 및 갈락토만난의 최대 농도는 각각 9.44 g/l 및 2.72 g/l 이었다. Sepharose CL-6B 젤 크로마토그래피와 다당체 성분 분석 결과, crude 갈락토만난의 조성은 주로 mannose (74.9%)와 galatose (24.1%)로 구성된 이종의 다당체인 것으로 밝혀졌다. *Trichoderma erinaceum* DG-312로부터 생산된 갈락토만난은 아세트산으로 처리한 쥐에서 체중(kg)에 대하여 100 또는 200 mg의 양으로 처리하였을 때 농도에 비례하여 현저한 항염증 활성을 갖는 것으로 나타났다. 갈락토만난의 항염증 활성을 나타내는 모세혈관 투과도(vascular permeability) 및 발작 반응(writhing response)의 저해 수치는 각각 60.6% 및 62.5%로 나타났다. 따라서 이런 갈락토만난의 항염증 및 발작반응 저해에 효과적인 특성을 이용한다면 치료 목적을 위한 의약품질 개발의 잠재성을 보여준다.