Enhancement of β -Glucan Content in the Cultivation of Cauliflower Mushroom (*Sparassis latifolia*) by Elicitation

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Abstract The effectiveness of three kinds of enzymes (chitinase, β -glucuronidase, and lysing enzyme complex), employed as elicitors to enhance the β -glucan content in the sawdust-based cultivation of cauliflower mushroom (*Sparassis latifolia*), was examined. The elicitors were applied to the cauliflower mushroom after primordium formation, by spraying the enzyme solutions at three different levels on the sawdust-based medium. Mycelial growth was fully accomplished by the treatments, but the metabolic process during the growth of fruiting bodies was affected. The application of a lysing enzyme resulted in an increase in the β -glucan concentration by up to 31% compared to that of the control. However, the treatment resulted in a decrease in mushroom yield, which necessitated the need to evaluate its economic efficiency. Although we still need to develop a more efficient way for using elicitors to enhance functional metabolites in mushroom cultivation, the results indicate that the elicitation technique can be applied in the cultivation of medicinal/edible mushrooms.

Keywords Elicitor, Fruit body, Medicinal, Metabolite, Sawdust-based media

Elicitors are physical or chemical factors that enhance or extend the production of desirable products by inducing biochemical changes in the cell culture of plants or microorganisms. Numerous publications have indicated the effects of elicitation on plant cell cultures since 1970s [1] and on microbial cultures in the 2000s [2-6]. Although elicitation has also been applied in the submerged culture of medicinal mushrooms [6] or in liquid cultures of several basidiomycetes [5], it has not been used in the practical cultivation of mushrooms. In this study, we established a hypothesis that elicitation can be applied in the cultivation of mushrooms, especially for the cultivation of edible/medicinal mushrooms.

Cauliflower mushroom (*Sparassis crispa* and/or *S. latifolia*) is an edible/medicinal mushroom which is now easily cultivable in Korea, using conifers [7, 8]. This mushroom was originally known as *Sparassis crispa*, but recently renamed as *S. latifolia* by Ryoo *et al.* [9]. Since the fruiting body of *S. crispa* contains a remarkably high content of β -glucan (43.6%), it shows good anti-tumor activities [10] and/or hematopoietic responses [11]. However, the productivity of the mushroom is largely dependent upon the kind of media and/or the strain used for the cultivation [12], which indicates that the functional materials may also vary by the condition of mushroom cultivation, including impacts of elicitation.

In this study, we adapted the concept of elicitation to the cultivation of cauliflower mushroom. The role of oligosaccharides as biotic elicitors for the enhanced production of antibiotics and enzymes has already been established [4, 5]. Thus, we wanted to examine whether the stimulation of fungal cell walls by biotic elicitors might result in an increase in the content of β -glucan in the cauliflower mushroom. Since the cell wall of fungal mycelium is mainly composed of chitins and acetyl-glucosamine [13], we used chitinase, β -glucuronidase, and a lysing enzyme complex to induce elicitation on the fungal cell wall. The effects of elicitors on the content of β -glucan, a key functional metabolite in cauliflower mushroom, were examined to determine the possibilities of adapting the elicitation concept for the cultivation of edible/medicinal mushrooms

Mycobiology 2014 March, 42(1): 41-45
<http://dx.doi.org/10.5941/MYCO.2014.42.1.41>
pISSN 1229-8093 • eISSN 2092-9323
© The Korean Society of Mycology

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Received October 1, 2013

Revised December 20, 2013

Accepted January 6, 2014

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such as *S. latifolia*.

MATERIALS AND METHODS

Cultivation of cauliflower mushroom on sawdust-based medium. A sawdust-based medium was used for the cultivation of cauliflower mushroom as reported by Park *et al.* [7]. The sawdust of conifers, barley flour, and sugar were mixed in a ratio of 80 : 20 : 3 (w/w/w) and the moisture content was adjusted to approximately 65%. Three kinds of conifers were used for the preparation of the sawdust-based media; *Larix kaempferi*, *Pinus koraiensis*, and *P. densiflora*. The pH of the mixture ranged from 4 to 4.5. The mixture was bottled in a 500-mL incubation bottle with the density of 0.72 g/cm³. We made a 1.5 cm in diameter hole with a depth of 5 cm in the center of the medium. The bottles were autoclaved for 90 min at 121°C.

We prepared the inoculum from a preserved strain isolated from a stand of *P. koraiensis* in Korea. It was cultured in potato dextrose broth for 3 wk to prepare it for use as a spawn. We inoculated 10 mL of liquid inoculum of *S. latifolia* into each bottle, and the amount of inoculum was 13 ± 1 mg dry weight. The bottles were incubated in a dark room at an ambient temperature of $23 \pm 1^\circ\text{C}$, with $60 \pm 5\%$ relative humidity. It took about 2 mo to fill a whole bottle with mycelium under the dark incubation. Every treatment had five replicates.

Application of elicitors. Since the cell culture usually targets the mass production of secondary metabolites, the elicitors are being applied right after the exponential growth stage, which may provide enough time for the growth of cells [6, 14]. Similarly, in this study, the elicitors were applied after the mycelial growth reached the exponential stage; we observed several primordia on the top of each sawdust-based medium filled with whitish mycelia. It was our aim that the treatments affect metabolic processes during the growth of fruiting bodies, while they do not affect the vegetative growth of mycelia.

The elicitors were prepared with three kinds of enzyme solutions; chitinase (5 units/L; Sigma-Aldrich Co., St. Louis, MO, USA), β -glucuronidase (25,000 units/L; Sigma-Aldrich Co.), and cell lysing enzyme (0.1 g/L; Sigma-Aldrich Co.). The enzyme solutions were applied in three levels; 10 mL, 20 mL, and 30 mL.

We applied the elicitors by spraying them directly onto the medium after opening the cap of each bottle. We tried to maintain the same incubation time for every medium. The average time needed to fill the bottle with mycelium was two months. This was also the ideal time for the *P. densiflora* medium to be moved into the fruiting room. However, the *L. kaempferi* medium was somewhat overmature at the time, while that of *P. koraiensis* was more or less immature.

The bottles were kept in a dark room for 24 hr after applying elicitors, and they were then moved into the

fruiting room, which was kept at an air temperature of $23 \pm 1^\circ\text{C}$, with $95 \pm 5\%$ relative humidity. About a month later, each bottle was harvested for the fruiting body. The yield was calculated by the total weight of fresh mushrooms and the weight of the upper part (pileus), which was considered to be the tradable goods. The lower part (stem) of the mushroom was not suitable for trading as an edible mushroom.

Assay for the glucan contents. A β -glucan assay kit (Megazyme International, Wicklow, Ireland) was used to determine total, α -, and β -glucan. The dried mushroom samples were milled to pass through a 0.5 mm screen by a centrifugal mill.

To measure the total glucan (α -glucan + β -glucan), 100 mg of sample was placed in a culture tube and 1.5 mL of concentrated HCl (37% v/v) was added. All of the tubes were placed in a water bath at 30°C for 45 min and were stirred on a vortex mixer every 15 min to ensure complete dissolution of the β -glucan. Each tube received 10 mL of distilled water, and the contents were stirred on a vortex mixer. They were incubated for 2 hr in a boiling water bath ($\sim 100^\circ\text{C}$). The tubes were cooled to room temperature, and received 10 mL of 2 N KOH. The contents of each tube were quantitatively transferred to a 100 mL volumetric flask using 200 mM sodium acetate buffer (pH 5.0) to wash the tube, and to adjust the volume. An aliquot of each suspension was separated by centrifuge at 14,000 rpm for 10 min. Aliquots of the centrifuged extract measuring 0.1 mL were transferred to glass test tubes. The tube contents were mixed with 0.1 mL of a mixture of exo-1,3- β -glucanase (20 U/mL) plus β -glucosidase (4 U/mL) in 200 mM sodium acetate buffer (pH 5.0), and were incubated at 40°C for 60 min. Subsequently, 3.0 mL of glucose oxidase/peroxidase mixture (GOPOD) was added to each tube and it was incubated at 40°C for 20 min. The absorbance of all solutions was measured at 510 nm against the reagent blank.

To quantify α -glucan, 100 mg of milled mushroom samples were suspended with 2 mL of 2 M KOH by stirring with a magnetic stirrer for about 20 min in an ice/water bath. Each tube was then given 8 mL of 1.2 M sodium acetate buffer (pH 3.8), and immediately treated with 0.2 mL of amyloglucosidase (1,630 U/mL) plus invertase (500 U/mL). All of the tubes were placed in a water bath at 40°C for 30 min with intermittent mixing on a vortex stirrer. These tubes were centrifuged at 14,000 rpm for 10 min. For these samples, the final volume in the tube was approximately 10.3 mL. Aliquots of supernatants measuring 0.1 mL were transferred into glass test tubes and 0.1 mL of sodium acetate buffer (200 mM, pH 5.0) plus 3.0 mL of GOPOD reagent were added and the tubes were incubated at 40°C for 20 min. Each absorbance measurement was determined at 510 nm against the reagent blank.

The quantity of β -glucan was calculated based on the amount of total glucan and α -glucan. That is, the subtraction

of the quantity of α -glucan from that of total glucan provided that of β -glucan. Each sample was analyzed in triplicate.

Statistical analysis. The mushroom yield and β -glucan content among the species and treatments were compared by ANOVA with SAS. The mean values of each observation were compared by a least significant difference test at the 5% level.

RESULTS AND DISCUSSION

Differences among the three kinds of sawdust-based media. The content of β -glucan in the *S. latifolia* harvested from our routine cultivation method was less than 40% (Fig. 1), which was substantially lower than that reported by Ohno *et al.* [10]. The content of β -glucan from the mushroom cultivated on the sawdust-based medium of *P. koraiensis* showed an even lower β -glucan content; less than 35%. This shows that the condition of production might affect the content of functional materials in the organism. This has made us seek a way to improve the content of functional material by applying a special stimulus such as an elicitor.

Responses against elicitation on the *L. kaempferi* sawdust-based medium. The content of β -glucan in the *S. latifolia* cultivated on the *L. kaempferi* sawdust-based media was significantly increased by the treatments with 30 mL of lysing enzyme and 10 mL of glucuronidase compared to that of the control. The content of β -glucan even reached up to 48.7% (Fig. 2). The contents of β -glucan increased 24.6% and 11.8% by the treatment of *S. latifolia* with 30 mL of lysing enzyme and 10 mL of glucuronidase, respectively. However, the yield showed a slight decrease when exposed to the treatment (Fig. 3), which demanded evaluation of the trade-off between yield and concentration of β -glucan. Although the total yield decreased 11.2% and 7.6% by the treatment of 30 mL of lysing enzyme and 10 mL of glucuronidase, respectively, the decrease was not significantly different in least significant

Fig. 1. Concentration of glucan in the fruit body of *Sparassis latifolia* cultivated on a sawdust-based medium from three kinds of conifers. The same letters on each bar indicate that the values were not significantly different by the least significant difference test at the 5% level ($n = 3$).

Fig. 2. Changes in glucan concentrations by the elicitor treatments in the cultivation of *Sparassis latifolia* on a *Larix kaempferi* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The same letters on each bar indicate that the values were not significantly different by the least significant difference test at the 5% level ($n = 3$, $p = 0.0066$).

Fig. 3. Changes in the mushroom yield by elicitor treatments in the cultivation of *Sparassis latifolia* on a *Larix kaempferi* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The same letters on each bar indicate that the values were not significantly different by the least significant difference test at the 5% level ($n = 5$; $p = 0.0193$ for total, 0.1206 for goods).

difference (LSD) test at the 5% level. In the case of the yield of goods (flowering parts), these results did not show statistically significant differences at the 5% level, although the treatments resulted in a 12.2% decrease and a 4.6% increase in the goods when 30 mL of lysing enzyme and 10 mL of glucuronidase, respectively, were applied. Thus, we recommend the treatments be used in the cultivation of the cauliflower mushroom in a *L. kaempferi* sawdust-based medium to enhance the content of β -glucan.

Responses against elicitation on the *P. koraiensis* sawdust-based medium. Although the contents of β -glucan in the *S. latifolia* cultivated on the *P. koraiensis* sawdust-based media were lower than those from the other media, the contents were significantly increased by the various enzyme treatments (Fig. 4). The contents of β -

Fig. 4. Changes in glucan concentrations by elicitor treatments in the cultivation of *Sparassis latifolia* on a *Pinus koraiensis* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The same letters on each bar indicate that the values were not significantly different by the least significant difference test at the 5% level ($n = 3$, $p = 0.0394$).

Fig. 5. Changes in the mushroom yield by elicitor treatments in the cultivation of *Sparassis latifolia* on a *Pinus koraiensis* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The values were not significantly different at the 5% level by ANOVA ($p = 0.4668$ for total, 0.5060 for goods).

glucan increased to 31.2% by the treatment with 10 mL of chitinase, 25.6% by 30 mL of glucuronidase, 22.2% by 20 mL of lysing enzyme, and 21.6% by 10 mL of glucuronidase. Furthermore, the decrease in yield was less than 10% compared to that in the control (Fig. 5) and had no statistically significant differences. This indicates that the treatments could be used in the cultivation of cauliflower mushrooms in a *P. koraiensis* sawdust-based medium to enhance the β-glucan content.

Responses against elicitation on the *P. densiflora* sawdust-based medium. The content of β-glucan in *S. latifolia* cultivated on the *P. densiflora* sawdust-based medium did not show any significant increase from the treatments. Although some treatments, such as 20 mL of lysing enzyme and 10 mL of glucuronidase, showed higher values than those of the control (Fig. 6), the LSD values were not significantly different at the 5% level. In addition, the yield of the mushrooms treated with 10 mL of glucuronidase decreased by 7.2% due to the treatment (Fig. 7). Thus,

Fig. 6. Changes in glucan concentrations by elicitor treatments in the cultivation of *Sparassis latifolia* on a *Pinus densiflora* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The values were not significantly different at the 5% level by ANOVA ($p = 0.2993$ for β-glucan).

Fig. 7. Changes in the mushroom yield by elicitor treatments in the cultivation of *Sparassis latifolia* on a *Pinus densiflora* sawdust-based medium. G, glucuronidase; Ly, lysing enzyme; Ch, chitinase. The numbers indicate the amount of each enzyme for each bottle in mL. The same letters on each bar indicate that the values were not significantly different by the least significant difference test at the 5% level ($n = 5$; $p = 0.0055$ for total, 0.0070 for goods).

we concluded that the treatments resulted in negligible improvement in the content of β-glucan in the cultivation of *S. latifolia* in the *P. densiflora* sawdust-based medium; hence was not considered an efficient treatment.

We examined whether or not elicitation could be applied to the sawdust-based cultivation of cauliflower mushrooms to enhance the β-glucan content in mushroom. Since the basal media resulted in differences in the content of β-glucan, we compared the productivity of each medium, one by one. Although there were several variations depending on the type of sawdust-based media, several enzyme treatments resulted in an increase in β-glucan concentration by up to 31% compared to that of the control. Thus, we are able to conclude that the stimulation of fungal cell wall by biotic elicitors may enhance the β-glucan concentration in cauliflower mushrooms. However, the treatments resulted in a decrease in mushroom yield, which demanded that we evaluate the economic efficiency of the treatment. In practice, we need to get more information to calculate the inputs

and outcomes more precisely in order to determine the true usefulness of this method of enhancing β -glucan content. The profitability is dependent upon a number of factors including the unit price of mushrooms, additional labor charges, and the price of enzymes. It is not easy to talk about profitability as the unit price of the mushroom in the trade market is variable. Thus, we can state that we have realized a possible method to adapt the concept of elicitation in the cultivation of medicinal/edible mushroom such as *S. latifolia* although we still need to develop a more efficient way to use elicitors to enhance functional metabolites in mushroom cultivation.

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