The mycelial growth and ligninolytic enzyme activity of cauliflower mushroom (Sparassis latifolia)

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ABSTRACT

We examined the activities of lignin-degrading enzymes of the mycelium of cauliflower mushroom (Sparassis latifolia). Three different strains of S. latifolia collected from several sites in Korea and one crossbred strain were cultured on potato dextrose broth (PDB) and Kirk’s medium in order to study the activities of their ligninolytic enzymes. Mycelial growth reached maximum levels between 14 and 21 days after inoculation and pH increased by 0.12 units over 35 days. Laccase activity began increasing after 14 days on both types of media. Manganese peroxidase (MnP) activity followed a trend similar to that of laccase on Kirk’s medium, but not on PDB. The activity of lignin peroxidase (LiP) differed from that of other enzymes; its activity decreased by half after 14 days on PDB but remained constant on Kirk’s medium over 35 days. The total protein concentration increased considerably after 14 days and peaked at 21 days on PDB. A similar maximum was attained on Kirk’s medium. In contrast, the residual glucose increased rapidly at 14 days on Kirk’s medium, while increasing gradually up to 28 days on PDB. This study indicates that S. latifolia is more similar to white rot fungi than to other brown rot fungi.

Introduction

Sparassis species are commonly associated with conifers and found on Abies holophylla, Pinus densiflora, Pinus koraiensis, and Larix kaempferi in northern temperate forests (Rua et al. 2009). They are classified as brown rot fungi and cause heart rot in living trees (Weir 1917; Gilbertson and Ryvarden, 1986, 1987; Wang et al. 2004). One of the most important features distinguishing brown rot from white rot fungi is lignin-degrading enzyme activity, which is exhibited only by the latter. Ligninolytic activity is assessed by colorimetric reactions of media with gum guaiac solution (Martin and Gilbertson 1976). The test results were negative for the production of extracellular oxidases for cultures of S. radicata, S. crispa, and their interspecies from Europe, Japan, and the US.

However, wood decay types have largely been determined by phylogenetic analysis (Hibbett and Donoghue 2001). For instance, Sparassis forms a monophyletic group with white rot fungi species. Thus, a closer examination of Sparassis is necessary in order to conclusively assign a wood decay type to this genus. The present study was undertaken in order to resolve this issue by examining the lignin-degrading enzymatic activities of laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP), as well as protein and residual glucose concentrations, in cultures of the cauliflower mushroom S. latifolia.

Materials and methods

Strains

Four S. latifolia strains were used in this study. Strains KFRI 700, KFRI 723, and KFRI 923 were collected in Korea, while KFRI 1750 was generated by crossbreeding KFRI 700 and KFRI 923 (Table 1). The four isolates were clearly identified as S. latifolia by taxonomic and molecular characteristics (Ryoo et al. 2013; Sou et al. 2013). For each strain, single spores were used for monokaryotic–monokaryotic (mon–mon) crosses (Uharte and Albertó 2009), in which two different haploid hyphae generate new dikaryotic hyphae. Crossbred mycelia produced clamp connections, and interactions between crossbred (KFRI 1750) and parent strains (KFRI 700 and KFRI 923) were examined in confronted cultures grown on potato dextrose agar (Difco) (Figure 1).

Ligninolytic enzyme activity assay

Sparassis latifolia cultures were grown in potato dextrose broth (PDB) and Kirk’s medium (Kirk et al. 1978). 70 mL test tubes each containing 25 mL of PDB and Kirk’s medium were autoclaved at 121 °C for 20 min and inoculated with agar plugs 4 mm in diameter cut from the actively growing margins of subcultures. Cultures were incubated at 23 °C under dark, stationary conditions for 7, 14, 21, 28, and 35 days.

Laccase activity was measured using 0.005 M ABTS (2,2-azinobis-[3-ethylbenzthiazoline-6-sulphonate]) as a substrate in 0.1 M sodium acetate buffer (pH 5.0) for a final volume of 500 μL, and the absorbance was measured at 420 nm (molar extinction coefficient ε = 36,000 M−1 cm−1); this and all other absorbance measurements were made with a UV spectrophotometer (Benchmark Plus, Bio-Rad). LiP activity was measured using 0.02 M veratryl (3,4-dimethoxybenzyl) alcohol as a substrate and 0.4 mM hydrogen peroxide in 0.05 M sodium tartrate buffer. An absorbance change at 310 nm (ε = 9,300 M−1 cm−1) was indicative of lignin enzymatic degradation (Vares et al. 1995). MnP activity was measured using 0.001 M guaiacol as a substrate in a solution of 0.001 M 0.001 M guaiacol as a substrate in a solution of 0.001 M H2O2 at 310 nm (ε = 26,000 M−1 cm−1).
manganese sulfate monohydrate and 0.001 M hydrogen peroxide in 0.5 M sodium tartrate buffer. The absorbance was measured at 525 nm ($\varepsilon = 121,000 \text{ M}^{-1}\text{cm}^{-1}$).

Protein concentration was measured with a protein assay reagent (Bio-Rad) using the Bradford method (Bradford 1976). Bovine serum albumin was used for the protein standard, and the absorbance was measured at 595 nm. The residual glucose was measured using the protocol described by Miller (1958), with a solution consisting of 10 g of 3-5-dinitrosalicylic acid, 2 g of phenol, 0.5 g of sodium sulfite, 200 g of potassium sodium tartrate, and 500 mL of 2% sodium hydroxide buffer per 1 L of distilled water. The absorbance was measured at 575 nm.

### Mycelial growth and pH

Mycelial growth was estimated from mycelial dry weights from samples recovered every 7 days over 5 weeks at 23 °C. The pH of the media was also measured during this interval.

### Results

#### Crossbred strain

Crossbred strain KFRI 1750 was originated from two parent strains KFRI 700 and KFRI 923. Clamp connections in Figure 1(A–B) represent the dikaryotic of crossbred strain. The presence of clamp connections in crossbred strain meant that success of the crossbreeding. Monokaryons originating from an isolated single spore of parent strains KFRI 700 and KFRI 923 are confirmed in Figure 1(C–D).

#### Ligninolytic enzyme activity

Laccase activity reached a peak at 14 days for cultures grown on both PDB and Kirk’s medium. Although there is little variability in the results from cultures grown on the same medium, laccase activity was higher by about 0.2591 units/mL when PDB was used (Figure 2). LiP activity decreased abruptly by half after 14 days on PDB and had variable levels when grown on Kirk’s medium over 35 days, while the activity of three of the strains increased continuously on both media (Figure 3). MnP activity showed trends similar to those observed for laccase activity, increasing after 14 days. The activities of all three ligninolytic enzymes were consistently higher for cultures grown on PDB than on Kirk’s medium (Figure 4).

#### Concentration of protein and glucose

Protein concentration increased significantly after 14 days and reached a peak at 21 days for cultures grown on PDB and Kirk’s medium, after which time the concentrations decreased. As with enzymatic activity, protein concentration in PDB cultures was generally higher than in cultures grown on Kirk’s medium (Figure 5).

In contrast, in cultures grown on Kirk’s medium, the residual glucose concentration increased rapidly between 14 and 28 days, while the increase was gradual over the first 28 days on PDB. In both cases, the residual glucose

### Table 1. Sparassis latifolia strains used in this study.

| Strain     | Year of collection | Host species   | Region of origin |
|------------|--------------------|----------------|------------------|
| KFRI 700   | 2005               | Larix kaempferi | Gyeonggi-do (Korea) |
| KFRI 723   | 2006               | Pinus koraiensis | Gyeonggi-do (Korea) |
| KFRI 923   | 2007               | Larix kaempferi | Jeollanam-do (Korea) |
| KFRI 1750  | Crossbred strain (KFRI 700 × KFRI 923) | | |

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Figure 1. Micrograph of mycelia from the crossbred strain KFRI 1750. Arrows indicate clamp connections, confirming that the new mycelia are dikaryotic, while the presence of septa confirms that the parent strains are monokaryotic. Clamp connections of KFRI 1750 (A–B), monokaryon originating from an isolated single spore of parent strain KFRI 700 (C), monokaryon originating from an isolated single spore of parent strain KFRI 923 (D).
Figure 2. Laccase activity in four strains of *S. latifolia* (KFRI 700, KFRI 723, KFRI 923, and KFRI 1750) grown on PDB (P) and Kirk’s medium (K) over 35 days. Means ± SE (*n* = 3).

Figure 3. LiP activity in four strains of *S. latifolia* (KFRI 700, KFRI 723, KFRI 923, and KFRI 1750) grown on PDB (P) and Kirk’s medium (K) over 35 days. Means ± SE (*n* = 3).

Figure 4. MnP activity in four strains of *S. latifolia* (KFRI 700, KFRI 723, KFRI 923, and KFRI 1750) grown on PDB (P) and Kirk’s medium (K) over 35 days. Means ± SE (*n* = 3).

Figure 5. Protein production in four strains of *S. latifolia* (KFRI 700, KFRI 723, KFRI 923, and KFRI 1750) grown on PDB (P) and Kirk’s medium (K) over 35 days. Means ± SE (*n* = 3).
concentration peaked at 28 days and rapidly declined thereafter (Figure 6).

**Mycelial biomass and pH**

Dry weight was used as an approximate measure of mycelial biomass. Similar results were observed for cultures grown on the two types of media; however, the biomass was approximately 0.04 mg higher when cultured on Kirk’s medium. Mycelial biomass reached maximum levels between 21 and 28 days after inoculation (Figure 7). pH increased slightly by about 0.12 units over 35 days on PDB; however, on Kirk’s medium, pH decreased by about 0.31 units over the same time course (Figure 8).

**Discussion**

Many studies have measured the lignin-degrading activity of *Sparassis* using various experimental protocols. In one study, extracellular oxidase was not detected when a spot test (Nobles 1965) was carried out (Martin and Gilbertson 1976). This test was conducted by placing a drop of guaiacum solution on a mycelial mat, followed by a 1 week incubation period. These investigators also used a modified version of Nobles’s method, in which cultures were incubated in medium containing gallic and tannic acid and gum guaiac solution over a 6 week period. Since they were mating conspecifics (*S. radicata* and *S. crispa* from Europe, Japan, and the US), the absence of lignin-degrading enzymatic activity was largely presumed. On this basis, the authors suggested...
that the mechanism of white rot fungi and tetrapolar mating systems were stimulated by brown rot fungi and bipolar mating systems. Several other tests for ligninolytic enzymatic activity in *Sparassis*, such as α-naphthol and guaiacol for laccase, p-cresol for tyrosinase, and pyrogallol for peroxidase followed by a 1 week incubation period also produced negative results (Stalpers 1978).

Nevertheless, the earlier explanations questioned by challenges to the conventional association between the mechanisms of decay caused by wood fungi, and the mating systems and substrate ranges (Hibbett and Donoghue 2001). Based on phylogenetic analyses, these authors inferred that the evolution of brown rot fungi preceded that of white rot fungi due to their exclusive use of conifers as substrate. In phylogenetic analyses based on differences in substrate selection between brown rot and white rot fungi, the genus *Sparassis* is included in the polyporoid clade, which includes wood decay fungi and is dominated by white rot fungi (Hibbett and Donoghue 2001). In analyses that are based on the correlation between mode of wood decay and substrate range, members of the genus *Sparassis* have ambiguous characteristics and are described as brown rot producers but are included in the same clade as white rot fungi. Therefore, it is important to consider not only phylogenetic and taxonomical characteristics, but also physiological and chemical properties in the classification of *Sparassis* species.

This study endeavored to measure the lignin-degrading activities of *S. latifolia* using a veratryl alcohol assay for LiP according to Kirk’s method (Kirk et al. 1978), in addition to laccase and MnP assays. The results revealed that *S. latifolia*, which is classified as a brown rot fungus, demonstrates the lignin-degrading activity that characterizes white rot fungi (Figures 2–4). Malt extract agar and cherry decoction agar media were previously used to assay activity (Martin and Gilbertson 1976; Stalpers 1978); therefore, PDB and Kirk’s medium were selected for this study. The laccase activity of the white rot fungus *Gerronema nemorale* examined under the same conditions was around 2–12 units/mL (Antonin et al. 2008), while for *S. latifolia* the value was 0.05–0.04 units/mL. In *G. nemorale*, MnP and LiP activities were 0.01–0.3 units/mL and 0.1–0.8 units/mL, respectively, while the values in *S. latifolia* were found to be 0.001–0.035 units/mL for MnP and 1–11 units/mL for LiP activity. The activities of laccase and MnP, but not of LiP, were higher in *G. nemorale* than in *S. latifolia*, although in *S. latifolia* LiP activity was higher than laccase and MnP activities. In previous studies, enzymatic activities in *Sparassis* were measured after 1 week incubation (Martin and Gilbertson 1976; Stalpers 1978); in the present study, the activities of laccase and LiP, as well as the concentrations of protein and residual glucose changed rapidly after 14 days, possibly due to physiological functions.

It is known from studies in *Cerrena unicolor* and *Pleurotus ostreatus* that media composition affects the activity of ligninolytic enzymes. The concentrations of peptone and wheat bran, the sources of nitrogen, were related to the activities of laccase and MnP (Elisashvili and Kachlishvili 2009; Ha 2013). The present study also revealed differences in activity of ligninolytic enzymes as a result of media composition. A more systematic study is needed in order to determine the reasons for these differences.

Ligninolytic activity was also detected in cultures of *Pleurotus ostreatus*; laccase and MnP were speculated to be produced as primary and secondary metabolites (Ha et al. 2001; Ha 2013). LiP activity peaked at 7 days of incubation; maximum laccase activity was observed at 21 days, while the maximum MnP activity was observed at 28 days. In contrast, in a study of ligninolytic activity of *Stereum ostrea*, the results were similar for all three enzymes (Praveen et al. 2011). When the carbohydrate polymer glucan and glucose were depleted, lignin-degrading enzymatic activity increased (Leathan 1985; Ha 2013). In general, residual glucose levels are relatively constant over time compared to parameters such as glucose and lignin depletion (Kirk et al. 1978). This was not substantiated by the results of this study. Initial glucose concentrations of PDB and Kirk’s medium were 20 g/L and 10 g/L, respectively. Residual glucose concentration decreased to values between 0.01 mg/L and 0.05 mg/L after 7 days of incubation, while at 28 days it was 0.3 mg/L (Figure 6). These results suggested that *Sparassis* fungi consumed the greatest amount of glucose during the first 7 days of incubation; thereafter, only infinitesimal amounts of glucose were consumed or secreted. Also, we could not find the relationship between dry weight and lignin-degrading enzymatic activity by glucose consumption and secretion, with dry weights of strains higher in Kirk’s medium but ligninolytic enzyme activities were lower. The metabolism of lignin and ligninolytic activity were too low to support growth (Kirk et al. 1978). Thus, as previously suggested, the relationship between ligninolytic activity, growth, and specific metabolic enzyme activities warrants further investigation.

**Conclusion**

This study was conducted to examine the characteristics of cauliflower mushroom (*S. latifolia*) and the activities of lignin-degrading enzymes. Three different collected strains and one crossbred strain of *S. latifolia* were cultured on PDB and Kirk’s medium to estimate the activities of ligninolytic enzymes laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP), as well as protein and residual glucose concentrations. As a result, differences in ligninolytic enzymatic activity were observed between cultures incubated in different media. Thus, *S. latifolia*, previously classified as a brown rot fungus, demonstrates the lignin-degrading activity characteristic of white rot fungi.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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