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Application of Enzymes to Taxonomy of *Ganoderma* Species

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Abstract—The genus *Ganoderma* is typical wood-rotting fungi and its fruiting body has been used as an important herb in oriental medicine. Recent research discovered antitumor components from *Ganoderma lucidum*. Various *Ganoderma* species are being cultivated in Korea. However, taxonomic system of the genus *Ganoderma* has been based mainly on the macromorphology of fruiting bodies and the ultrastructural characteristics of basidiospores. Since there are similar characteristics in *Ganoderma* mycelia grown on the same artificial media, it is suggested that the compatibility of the fungi by di-mon mating be used as an aid to determine the identity of species in addition to the conventional characterization. In this study, we examined physiological and genetical properties such as growth temperature, pH, compatibility and enzyme or protein patterns of laccase, esterase and cellular proteins of *G. lucidum* RZ, *G. tsugae* and *Ganoderma* species cultivated in Korea by electrophoresis for characterization of the isolates. We found that compatibility test and isozyme patterns of laccase and esterase of the mycelia could be used for the differentiation of the isolates. These results showed that *Ganoderma* species cultivated in Korea is genetically similar to *G. lucidum* but physiologically closer to *G. tsugae* than to *G. lucidum*.

Keywords—*Ganoderma lucidum* • electrophoresis • laccase isozymes • esterase isozymes • *Ganoderma tsugae*

The *Ganoderma* species are typical wood-rotting fungi that cause white rots of a wide variety of trees and timber on a world-wide basis. The fruiting bodies of *Ganoderma* have been used as an important herb to cure various human diseases sush as hepatitis, hypertension, hypercholesterolemia and gastric cancer. Recent research discovered antitumor components of *Ganoderma lucidum.*

The genus *Ganoderma* was established by Karsten in 1881 with *G. lucidum* (W. Curt.: Fr.) Karsten as the only species. There are some intensive works on the taxonomy of the genus[1~4] based on brightness of the pileus and the morphology of the basidiospores. Nobles provided 11 characteristic key patterns and descriptions bssed on cultural information on 126 basidiomycetes that decay woods[5]. The descriptions of the cultural characteristics of *Ganoderma* species are very similar to each other. In addition, fruiting bodies of *G. lucidum* can be morphologically variable depending upon the envir-

onmental conditions, while isolates from such fruiting bodies are interfertile[2]. Basidiocarps of the *G. lucidum* species may also have similar microscopic characteristics. Although basidiospores of these fungi can be distinguished, natural variation and similarities in structure limit their sole use in the identification of the species. Nobles first reported distinct differences between *G. lucidum* and *G. tsugae* based on cultural observation.

Bazallo and Wright found that *G. lucidum* is a complex of very similar species[6] and Nobles suggested that interfertility tests be used as an aid to determine the identity of species[5]. In 1930, Buller first reported the dikaryotization of homokaryons. Quintanilha, in 1937, named this mode of dikaryotization as "the Buller phenomenon" or di-mon matings. It was established that hyphal fusions occurred between homokaryons and dikaryons and that nuclei could be transferred to the former. The regular and rapid dikaryotization of homokaryons based on the same species. Homokaryons of *Ganoderma* species, however, have been difficult to obtain because of low percentage or total lack of basidiospore germinaton. Adaskaveg and Gilbertson suggested that future species designations in the *G. lucidum* complex be considered valid only after cultural and interfertility studies are conducted[2].

Many basidiomycetes are known to produce multiple electrophoretic forms of intra and extracellular laccase (E.C. 1. 10. 3. 2) as revealed by polyacrylamide gel electrophoresis (PAGE) or isoelectric focusing[7,8]. Fungal laccases are extracellular phenol oxidases specified by their ability to oxidize monophenols, *o*- and *p*-diphenols, aminophenols and diaminoaromatic compounds using molecular oxygen. These enzymes are fungal specific and may play important roles in lignin degradation and/or detoxification of lignin degradation products. The laccase of *Aspergillus nidulans* appears during its conidiation and is responsible for a step in the synthesis of conidium pigment[9]. In the basidiomycete *Schizophyllum commune* laccase activity is positively correlated with the ability of monokaryons to fruit and with the stage of fruiting development in dikaryotic cultures[10,11]. During the growth of *Agaricus bisporus*, extracellular laccase activity is also increased and then declined rapidly at the time of fruiting[12].

Blaich and Esser found that the multiple forms of laccase enzymes were taxonomically useful at the species level, but not useful for higher-order groups[13]. Kerrigan and Ross reported the potential utility of extracellular laccase PAGE-separation patterns, zymograms, as markers for the delimitation of taxa and the reconstruction of phylogenetic relationships in the genus *Agaricus*[14]. Hseu and Wang using syringaldazine, classified three types of *Ganoderma* according to the change of laccase activity[15] and analyzed the laccase isozymes by electrophoresis suggesting that there is the feasibility of using the laccase zymograms to identify different species and to compare different isolates of *G. lucidum*[16]. Isozyme patterns of esterase and protein from 16 isolates of *Ganoderma lucidum* were also observed by electrophoresis for characterization of the isolates[17].

The objectives of this study are to compare isolates on the basis of macro- and microscopic cultural morphology and temperature relationships, and to examine laccase, esterase and protein isozyme patterns that might provide a more specific methods for clarifying the taxonomy of cultures of *G. lucidum*.

Materials and Methods

1. Fungal Strains

Strains of *Ganoderma lucidum* RZ and *Gano-*

derma tsugae G10 were provided by Dr. R. S. Hseu of National Taiwan University, Taipei. *Ganoderma* species cultivated in Korea was used as 'sample strain' in this study.

2. Effects of Temperature

Optimal growth temperature ranges were determined by growing isolates for two weeks on 2% malt extract agar(MEA) medium at various temperatures ranging from 15 to 37°C. The experiment had three replications and was repeated twice.

3. Compatibility Test

Compatibility tests were conducted with isolates in all possible combinations. For this, 5mm diameters inoculum plugs were cut from 7-day cultures and placed 2 cm apart on the same plate. The inoculated plates were incubated for at least 14 days at 30°C and then examined macroscopically for reaction zones and then microscopically for clamp connection using 1% phloxine and 4% KOH as mounting media. Compatibility crosses had three replications and were repeated twice.

4. Electrophoretic Patterns of Laccase Isozymes

Enzyme Production and Assay—Laccase samples were prepared by inoculation of a 5mm plug of a stock culture into 30ml of a liquid medium. After sufficient stationary incubation at 30°C, 0.6 ml of the medium was taken out aseptically every 2~3 days. Laccase activity was determined by measuring optical density at 525 nm with syringaldazine as substrate. The reaction mixture consisted of 0.6 ml of 0.4 mM syringaldazine solution, 2.2 ml of 0.2 M citrate-phosphate buffer (pH 5.4) and 0.2 ml of sample. The reaction was carried out at 32°C for 15 minutes.

Enzyme Preparation for PAGE—After stationary incubation for 2 weeks at 30°C, mycelia were removed from the culture broth by filtration. The filtrate was used as the source of crude enzyme. Samples were mixed with sample buffer (reservoir buffer containing 10% sucrose plus a trace of bromophenol blue). Volumes loaded were 40~60 μl/lane.

Electrophoresis for the Separation of Laccase Isozyme—Electrophoresis was carried out on vertical polyacrylamide slab gels measuring 9×8 cm, prepared with 4.0% stacking and 8.5% resolving gel concentrations under nondenaturing condition using buffers (Table I) modified from those of Laemmli[18]. Separation was done at 100~160V for 6~7 hours.

Table I. Buffers for extracellular laccase

Stock solution	pH	Recipe
Stacking gel buffer	6.8	0.5M Tris, 0.5M HCl
Resolving gel buffer	8.8	1.5M Tris, 0.5M HCl
Reservoir buffer	8.3	0.25M Tris, 0.92M Glycine

* The solution is filtered through Whatman No. 1 filter and stored at 4°C.

Gel Staining—Bands of protein with laccase activity were visualized in gels by immersion in a 0.4 mM syringaldazine solution buffered to pH 5.4 with 0.2 M citrate-phosphate buffer for 20 min at 32°C according to the method of Bollag[19].

5. Electrophoretic Patterns of Esterase and Protein

Mycelial Growth and Enzyme Extraction—Fungi grown on liquid medium for 2 weeks were harvested and freeze-dried. They were ground into a fine powder with a mortar and the same volume of the 0.1 M Tris-HCl buffer (pH 7.5) as that of ground mycelia was added. The mixture was centrifugated for 30 min at 12,000×g and the supernatant was used as a sample.

Electrophoresis for the Separation of Esterase Isozyme and Proteins—A high pH discontinuous system using nondenaturing polyacrylamide slab gel was employed. Stacking and resolving gel

Table II. Buffer system for esterase and protein

Stock solution	pH	Recipe
Stacking gel buffer	6.90	158 mM Tris, 289 mM HCl
Resolving gel buffer	8.48	947 mM Tris, 289 mM HCl
Lower tank buffer	7.49	63 mM Tris, 50 mM HCl
Upper tank buffer	8.89	37.6 mM Tris, 40 mM HCl

* The solution was filtered through Whatman No. 1 filter and stored at 4°C.

concentrations were 4.0 and 10.0% respectively under nondenaturing condition using modified Laemmli's buffer[20] (Table II). Each 60 μl of the sample was applied to the gel and electrophoresed at 80~160 V for 6~7 hours.

Gel Staining—a) Esterase isozyme: The gel was removed from the glass and followed the protocol supplied from the company (Sigma Chem. Co., U.S.A.) for the detection of esterase isozyme pattern. In short, the gel was fixed with citrate-acetate-methanol fixative solution (18 ml of 38.3 mM citrate buffer, pH 5.4; 27 ml of acetone and 5 ml of absolute methanol) for 30 seconds at room temperature, washed the gel twice with distilled water and air-dried for 20 min. The gel was finally placed in the developing solution (20mg α-naphthyl acetate, 2 ml ethylene glycol monoethyl ether, 10 mg fast blue RR salt and 50 ml of 20 mM tris-HCl buffer, pH 7.6) for 30 min at 37°C until the bands appeared clearly. b) Protein: The gel was stained in 0.25 % coomassie brilliant blue (Bio-Rad) dissolved in d.w. for an hour and destained in 5% methanol containing 7% acetic acid for 12 hr.

Results

1. Cultural Characteristics

Macroscopic Characteristics and Temperature Effect—Macroscopic cultural characteristics of *Ganoderma lucidum* and *G. tsugae* were illustrated by Adaskaveg and Gilbertson[2]. Mats of *G. lucidum* had an even margin and were felty in texture and white in color. Those of *G. tsugae* and the sample also had an even margin but were felty to floccose in texture and white to pale yellow in color. Older cultures of *G. tsugae* and the sample strain usually produced an undulating surface in pigmented regions that buckled or distorted the agar. Temperature studies indicated different growth rates and optimal temperature ranges for the isolates (Fig. 1). *G. lucidum* had an optimal temperature of 30°C with a growth rate of 4.2 mm/day. *G. tsugae* and the sample strain had an optimal temperature range of 20~25°C, with growth rates of 6.1~6.2 mm/day and 4.3~4.5 mm/day, respectively.

Microscopic Observations—Conspicuous clamp connections were observed in all the species on their generative hyphae. Hyphae were stained reddish pink when treated with KOH and phloxine. *G. lucidum* produced no binding hyphae, Bovista-hyphae, as previously described[22].

Compatibility Test—Two macroscopic reactions were observed: compatible (*G. lucidum*–G.

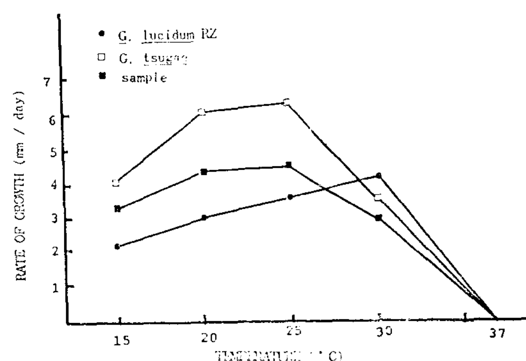

Fig. 1. Growth rates of *G. lucidum* RZ, *G. tsugae* and sample at temperature 15~37°C

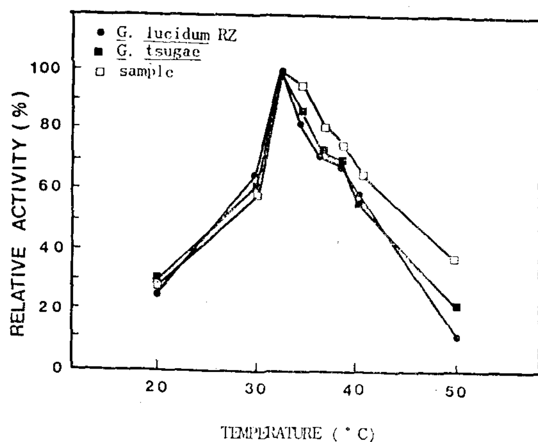

Fig. 2. Effect of temperature on the enzyme activity The reaction mixture consisted of 2.8ml of 0.8mM syringaldazine and 0.2ml of sample. The reaction was carried out for 15 min at the indicated temperature.

lucidum, *G. tsugae*-*G. tsugae*, the sample strain-the sample strain and *G. lucidum*-the sample strain) and incompatible (*G. tsugae*-the sample strain). Each pairing was further examined for clamp connections microscopically to see genetic relationship. Compatible pairing produced abundant clamp connections on their generative hyphae in 3~4 weeks indicating that they were genetically close and thus mated each other. On the contrary, incompatible pairing produced no clamps.

2. Enzymatic Properties of Extracellular Laccase

Effects of Temperature on Extracellular Laccase Activity—The effects of temperature on fungal laccase activity was examined with 0.2 M citrate-phosphate buffer (pH 5.4) to find the best conditions for the enzyme assay. The enzymatic reactions were carried out for 15 min at various temperatures. As shown in Fig. 2, the enzyme isolated from all three fungi was active over the temperature range of 30° to 40°C, with maximum activity at 32°C.

Effects of pH on Extracellular Laccase Activity—Various values of pH were examined with 0.2 M citrate-phosphate buffer (pH 5.0~6.0) to find the best conditions for this enzyme assay. The laccase from all three fungi was active over the pH range of 5.0 to 6.0 and had the maximum activity at pH 5.4 (Fig. 3). The enzyme activity decreased gradually above pH 5.4.

Relationship Between Extracellular Laccase Activity and Growth Rate of Fungi—After stationary incubation for 7 days at 30°C, 0.6

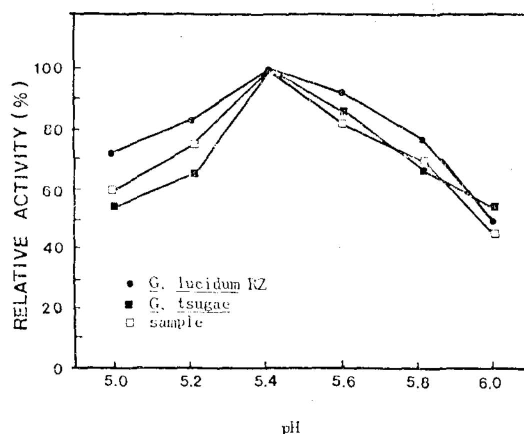

Fig. 3. Effect of pH on the enzyme activity
The reaction mixture consisted of 0.6ml of 0.4 mM syringaldazine, 2.2 ml of 0.2 M citrate-phosphate buffer solution and 0.2 ml of sample. The reaction was carried out at 32°C for 15 min.

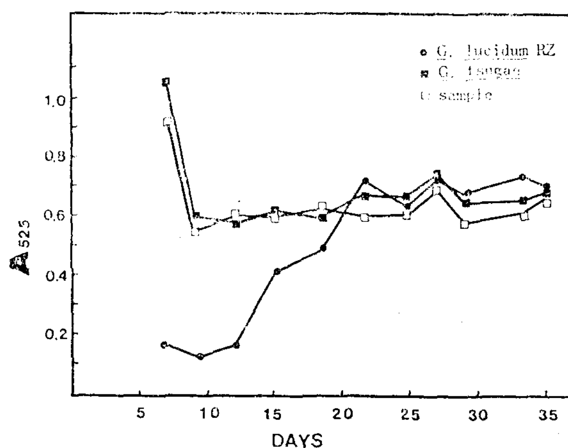

Fig. 4. Relationship between extracellular laccase activity and growth of *G. lucidum* RZ, *G. tsugae* and sample

ml of the medium was taken out every 2～3 days and the laccase activity was measured. The fungi investigated released considerable amounts of laccase into the growth medium and showed constant enzyme activity except *G. lucidum*. Relationship between laccase activity and growth of fungi is shown in Fig. 4. *G. tsugae* and sample strain released high concentration of laccase from 7th day and kept almost steady state. On the contrary, *G. lucidum* produced low concentration of laccase into the culture medium at the beginning, began to increase enzyme release and had almost the same amount on 20th day.

3. Electrophoretic Patterns of Isozymes

Extracellular Laccase and Esterase—Electrophoresis of extracellular laccases gave three isozyme bands in *G. lucidum* and one in *G. tsugae* and the sample strain, respectively, indicating that the sample strain is closer to *G. tsugae*. Extracellular laccase production was analyzed by SDS-PAGE at various growth stages of *G. lucidum*. Two laccase isozyme bands appeared strongly on 7th day, began to diminish after that day and completely disappeared on 28th day. An additional laccase isozyme band began to appear on 14th day and gradually reduced after that day. The band pattern was age-dependent.

Isozyme patterns of esterase and protein of *Ganoderma* species were also examined by electrophoresis for characterization of isolates. The esterase isozyme and protein patterns of the sample strain were similar to those of *G. tsugae* in the 8.5% SDS-PAGE analysis.

Discussion

Taxonomy of *Ganoderma* species was studied in this paper to clarify the species of *Ganoderma* that has been widely used in Korea. For this purpose, cultural morphology, growth temperature and isozyme patterns were studied by comparing with those of two standard *Ganoderma* species, *G. lucidum* RZ and *G. tsugae* G10. Distinctive macroscopic cultural characteristics of the isolates studied include growth margin, mat texture and mat color on MEA. Macroscopic cultural descriptions of *G. lucidum* and *G. tsugae* were as described by Nobles[5]. Temperature studies showed that *G. lucidum* had an optimal temperature of 30°C, whereas *G. tsugae* and the sample had an optimal temperature range of 20～25°C. In addition, the sample had a similar temperature curve to that of *G. tsugae*. *G. lucidum* can be differentiated from the other two isolates on the basis of macroscopic cultural characteristics and temperature relationships.

Compatibility test that examined all crosses microscopically for clamp connections and macroscopically reaction zones showed that four pairings, except one, were compatible. This compatibility test is based on their genetical background, thus compatible pairing has similar genetic background each other. Since the sample strain showed complete compatibility with *G. lucidum*, not with *G. tsugae*, the sample strain was closer to *G. lucidum* than to *G. tsugae* in genetic criteria.

The extracellular laccase activity in the culture medium of *Ganoderma* species was measured. The fungi investigated released considerable amounts of laccase into the growth medium and showed constant enzyme activity during the growth, indicating that laccase is generally present in culture broth. The optimal temperature of the enzyme reaction was 32°C, higher than that of laccases from *Lentinus edodes*[20] and *Schizophyllum commune*[8]. Optimum pH measurement for syringaldazine as substrate is in agreement with the results reported by Bollag and Leonowicz[19]. Relationship between laccase activity and growth of fungi was investigated and the results indicate that *Ganoderma* species may be

classified according to the change of laccase activity. In that experiment, the sample had an activity curve similar to that of *G. tsugae*.

The extracellular laccases liberated into liquid culture medium are useful for systematic taxonomy because they produce relatively simple patterns of bands which appear to be reproducible and largely invariant within species. To determine the time of optimal phenotypic expression, electrophoresis of sequentially drawn samples of *G. lucidum* culture broth, after sufficient stationary incubation, was carried out. Because identical band patterns were observed in the 2∼3 week-old samples, though the pattern of bands was age-dependent, extracellular laccases were isolated from the culture filtrates at that time.

Comparison of the isozyme band patterns of laccase showed that *G. tsugae* and the sample did not show any electrophoretic heterogeneity as similar bands were observed. *G. lucidum* differed in this respect. It had two additional bands of lower electrophoretic mobility. The esterase patterns of the mycelia could differentiate the isolates and the band patterns of protein in the extracts of each fungus were also different. Wong and Raper[21] have shown that in *S. commune* differences in protein patterns existed even among the homokaryotic and dikaryotic mycelia and the protein profiles of such widely divergent stages such as basidiocarps or mycelia in *A. bisporus* were different to a considerable degree when studied by electrophoresis[22]. Thus the sample strain was genetically similar to *G. lucidum*, but physiologically similar to *G. tsugae*. Further taxonomical studies are required to clarify *Ganoderma* species and are in progress.

Conclusions

Cultural morphology and temperature relationships of the mycelia of *Ganoderma* species provided information necessary to distinguish the isolates. *G. tsugae* and the sample showed nearly identical cultural characteristics and *G. lucidum* was differentiated from the other two isolates. The fungi studied had the characteristic patterns of laccase and esterase for each species. The laccase and esterase isozyme patterns of the mycelia could differentiate the isolates. The results show that the *Ganoderma* species examined in the study is closely related to *G. tsugae*.

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