ENZYMES OF STRAINS OF PLEUROTUS SPECIES (BASIDIOMYCETES) COMPARED BY ELECTROPHORESIS

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Four kinds of electrophoresis were used to characterize enzymes in 11 isolates from six species in the genus Pleurotus (Fr.) Quel: Pleurotus ostreatus, P. pulmonarius, P. spodoleucus, P. salmoneostramineus, P. cornucopiae, and P. cystidiosus. They were agarose isoelectric focusing (IEF), polyacrylamide IEF, 7.5% polyacrylamide gel electrophoresis (PAGE) and 4–15% gradient PAGE. Ultimately, the 4–15% polyacrylamide gel-slab electrophoresis was found to be most suitable for our study in numerically analyzing the patterns of seven enzymes. The enzymes were 6-phosphogluconate dehydrogenase (EC 1.1.1.41 6PGDH), malate dehydrogenase (EC 1.1.1.37 MDH), lactate dehydrogenase (EC 1.1.1.27 LDH), glutamate dehydrogenase (EC 1.4.1.4 GDH), glucose-6-phosphate dehydrogenase (EC 1.1.1.49 G6PDH), alcohol dehydrogenase (EC 1.1.1.1 ADH), and esterase (EC 3.1.1.1 Est). The isolates were compared by using an index of similarity (Sm) based on relative mobilities (Rm) of the enzyme bands. By this method, the isolates were reasonably grouped as species. Based on the Sm values, as well as intercompatibilities and morphological characters, the relationships between P. ostreatus, P. spodoleucus and P. pulmonarius were analyzed.

Since the first description of gel electrophoresis patterns (zymograph) of 14 enzymatic activities in the cell extracts of Schizophyllum commune(23), several reports on isozyme patterns of higher basidiomycetes have appeared in two areas, one with the aim of finding changes in isozyme patterns which might have taken place during the morphogenesis of these microorganisms(5, 11, 15, 16, 18, 22), and the other to identify or classify the basidiomycetes(2, 10, 14, 17, 19). However, the interpretation of the zymographic patterns have been descriptive or diagrammatic,

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lacking an objective parameter.

With yeast on the other hand, numerical analysis of zymographic patterns have long been undertaken (6, 20, 24).

Six defined species belonging to the genus *Pleurotus* (Fr.) Quél are found in Japan according to Imazeki et al. (7). They are *Pleurotus ostreatus* (Jacq.:Fr.) Kummer, *Pleurotus pulmonarius* (Fr.) Quél, *Pleurotus cornucopiae* (Paulet) Rolland var. *citrinopileus*, *Pleurotus salmonoeostamineus* L. Vass, *Pleurotus cystidiosus* O. K. Miller and *Pleurotus dryinus* (Pers.: Fr.). There is also one more *Pleurotus* sp. in Japan, called *Pleurotus spodoleucus* Imaz. & Toki which is not clearly defined in the literature. It is indistinguishable from *P. ostreatus* morphologically, but it still remains in doubt whether it should be defined as an independent species.

Since classification of the *Pleurotus* has been based mainly on morphological observations, it is difficult to distinguish strains whose fruiting bodies are easily affected by environmental factors (1).

It was the purpose of this study to provide objective parameters besides morphology and sexuality, for discriminating *Pleurotus* species by numerical analysis of zymographic patterns.

**MATERIALS AND METHODS**

*Organisms.* *Pleurotus ostreatus* KH37, *Pleurotus pulmonarius* KA01, four isolates (No. 1–4) of *Pleurotus cornucopiae* and two isolates (No. 1, 2) of *Pleurotus spodoleucus* are commercially available strains provided to us by the Laboratories of edible mushrooms Kawamura K. K. Their origins are listed in Table 1. *Pleurotus cystidiosus* IFO 9379, and *Pleurotus salmonoeostamineus* IFO 9573 were purchased from the Institute for Fermentation, Osaka. *Pleurotus ostreatus* which is now deposited in the American Type Culture Collection as ATCC 60691 was from the Nagano Vegetable and Ornamental Experimental Station. All of the strains are dikaryons and proven to be fertile in our laboratory.

*Cultural conditions.* The strains were maintained serially on potato dextrose agar (PDA).

| Table 1. Origins of isolates of *Pleurotus* species provided by Kawamura K. K. |
|-----------------------------|----------------------------------|
| **Species** | **Origin** |
| *Pleurotus cornucopiae* No. 1, 2, 3, 4<sup>a</sup> | isolated in 1968 from sporophores collected in fields of Hokkaido Pref. |
| *Pleurotus ostreatus* KH37 | isolated in 1982 from sporophores collected in fields of Aomori Pref. |
| *Pleurotus pulmonarius* KA01<sup>b</sup> | collected from commercial sporophores in Taiwan |
| *Pleurotus spodoleucus* No. 1, 2<sup>a</sup> | Isolated in 1968 from sporophores collected in fields of Yamagata Pref. |

<sup>a</sup> Different strain numbers designate that they were isolated from different sporophore tissues.

<sup>b</sup> Photographs of fruitbodies and dried samples were sent to Dr. Tsuguo Hongo and identified as *Pleurotus pulmonarius*. 


agar (Nissui) slants. To extract buffer-soluble proteins, they were inoculated in 500-ml Erlenmeyer flask containing 200 ml of liquid medium of the following composition: D-glucose, 10 g; yeast extract, 0.5 g; peptone, 0.5 g; KH$_2$PO$_4$, 0.2 g; MgSO$_4$·7H$_2$O, 0.5 g; CaCl$_2$·2H$_2$O, 0.1 g; 4 ml of minor elements in solution to give in the final solution, FeCl$_2$·6H$_2$O, 2 mg; MnCl$_2$·4H$_2$O, 1.4 mg; ZnCl$_2$, 0.8 mg; CuSO$_4$·5H$_2$O, 0.2 mg per liter. They were grown at 24°C static for 20 to 30 days.

Crossing experiment. Sporophores were grown on sawdust-rice bran medium in 250 ml glass bottles for morphological observations and to isolate monokaryons. Pairings between monokaryons were made by placing small pieces of inoculum 1 cm apart on malt extract agar in 9 cm Petri-dishes. Then the junction and periphery of the paired colonies were checked for the presence of clamp connections.

Preparation of buffer-soluble proteins. Mycelium from the liquid medium was harvested by suction filtration on a Buchner funnel. The harvested mycelia were rinsed three times with distilled water and once in 5 mM phosphate buffer (pH7.0) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF). The mycelia were then ground on ice with acid-washed sea sand in the phosphate buffer. The homogenate was centrifuged for 1 h at 27,000 x g (Hitachi RPRW20). The resulting supernatant was either used immediately or stored for use later at –20°C as 50% glycerol suspension. The total soluble protein concentration in the supernatant was estimated by the Bradford method (3).

Electrophoresis Agarose IEF: IEF was performed as written in the Pharmacia catalogue. Molten 1% agarose with 12% sorbitol and Pharmalyte (pH 3–10) were cast between two glass plates to make 90 x 110 x 1 mm gel-slabs. Samples were applied on filter paper strips. For the anode, 1 M NaOH and for the cathode, 0.05 M H$_2$SO$_4$ was used. Constant power (SW) was applied for 1.5 h.

PAG IEF: 0.5% PAG (110 x 110 x 0.5 mm, pH 3–10) was prepared on Gel-support (Atto) to avoid breaking the gel during the staining procedure. For the anode, 1 M NaOH and for the cathode, 0.04 M aspartic acid was used. Both IEFs were performed at 4°C on a flat bed apparatus (KS-8300 FSE, Marysol Ind. Co., Ltd.).

7.5% PAGE: 7.5% acrylamide gel slab (100 x 140 x 1 mm) was prepared with the apparatus (ATTO SJ-1060 SG). The running gel was 7.5% acrylamide in Tris-HCl buffer (pH 8.9) and electrophoresis was carried out at 4°C in Tris-glycine buffer (pH 8.3), 100V, until the dye front reached the edge of the gel.

4–15% PAGE: Same as 7.5% PAGE except 4–15% gradient polyacrylamide gel (84 (W) x 94 (H) x 1.0 mm, 12 wells) was purchased from Daiichi Pure Chemicals Co., Ltd. 30 mA constant current was applied for about 2 h.

Staining of enzyme activities. Immediately after the electrophoresis, the enzymes were visualized in the gel by the Yamazaki method (25), except that 0.05 M phosphate buffer was used instead of Tris-HCl. Briefly, dehydrogenases were stained by incubating the gel at either room temperature or at 37°C, in the reaction mixture containing 20 mg of substrate for each enzyme, 50 mg NAD or 20 mg NADP, 4 mg Phenazine methosulfate and 30 mg Tetrinitrotetrazolium Blue in 100 ml of 0.05 M.
phosphate buffer (pH 7.0). Esterase was stained according to the method of (9) in solution containing 2 ml of 1% α-naphthyl acetate dissolved in 50% aqueous acetone and 100 mg fast blue BB salt in 100 ml 0.05 M phosphate buffer (pH 7.0). After staining, the agarose gels were dried on filter paper. All the other gels were stored in zip-lock plastic bags without drying.

The relative electrophoretic mobilities (Rm) of PAGE were calculated between the bromophenol blue dye front and the visualized enzyme activity bands.

**Calculation of similarities.** To compare the strains in terms of all seven enzymes, an estimate of similarities was calculated using a Dice-type coefficient (21) defined as: \( S(\%) = \left[ \frac{2N_{AB}}{(N_A + N_B)} \right] \times 100 \), where \( S(\%) \): similarity value for each enzyme, \( N_{AB} \): no. of bands with same Rm; \( N_A \) and \( N_B \): no. of bands of strains A and B, respectively. The mean value of the seven \( S \) values was presented as the similarity index (Sm) between strains A and B. A dendrogram was made according to a single linkage procedure (8).

**Chemicals.** The agarose used for IEF was from Sigma No. A-4905. Pharmalyte 3–10 was from Pharmacia Fine Chemicals. Phenazine Methosulfate and Tetranitrotetrazolium Blue were from Wako Pure Chemical Industries, Ltd. NAD and NADP were purchased from Oriental Yeast Co., Ltd.

All the other reagents were of the finest grade available.

**RESULTS**

**Preparation of buffer-soluble proteins**

Concentration of the buffer soluble proteins of the mycelial extract ranged from 1.7 to 3.4 mg/ml except for *P. cystidiosus* IFO 9379 which was 0.6 mg/ml, because of its poor growth in the medium. The liquid medium might not have been appropriate for its growth. Depending on the protein concentration of the strains, 10 to 25 µl of the mycelial extract containing 10 µg of protein was applied to the

![Image of agarose gels](image)

Fig. 1. Isoelectric focusing of (a) MDH, (b) 6PGDH, (c) G6PDH on agarose gels.
Fig. 2. Diagram of isoelectric focusing of enzymes on PAG. 1. *P. cystidiosus* IFO 9379, 2. *P. salmoneostramineus*, 3. *P. pulmonarius* KA01, 4. *P. spodoleucus* No. 1, 5. *P. ostreatus* KH37, 6. *P. cornucopiae* No. 4.
Results of the four kinds of electrophoresis

Agarose IEF: Although agarose is more easier to handle than acrylamide, it did not necessarily offer higher resolution (Fig. 1) than PAG. LDH, 6PGDH, G6PDH, MDH and hexokinase could be stained in the gel. Due to the low pro-
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Protein concentration, the activity bands of *P. cystidiosus* IFO 9379 could be seen only with MDH.

**PAG IEF**: The results are diagrammed in Fig. 2. They gave high resolution of the dehydrogenases, but ADH and LDH lost their activity during the electrophoresis. The same phenomena were observed with 7.5% PAGE. Also, the activity bands of Est (Fig. 3) were too numerous and the MDH were broad for calculating similarity values without the aid of densitometer scanning. Therefore, we considered this method to be impractical for the numerical analysis in our study.

**7.5% PAGE**: ADH, LDH, 6PGDH, G6PDH and MDH were stained. The activity bands of ADH and LDH of *P. ostreatus* and *P. spodoleucus* did not appear in the gel just as in PAG IEF.

**4-15% gradient PAGE**: This was found to be most effective for preparing the enzyme patterns necessary for this study (Fig. 4). The activities of the seven enzymes of all the strains could be observed. It offered not only the best results in reproducibility but also higher resolution than the non-gradient PAGE system.

To compare the four electrophoretic methods, an example of MDH is shown in Fig. 5.

The $R_m$ values for activity bands of ADH, G6PDH, 6PGDH, LDH, GDH, MDH, and esterase in 4–15% PAGE are presented in Table 2. The $R_m$ values of those bands which were very broad or faint were neither presented in Table 2 nor included in calculating the similarity indexes.

**Similarities between the species**

The similarity index ($S_m$) calculated according to the formula (MATERIALS AND METHODS) are presented in Table 3. Those which were compatible with each other were shown to have $S_m$s larger than 66%.
Table 2. Relative mobilities (Rm) of bands with activity of seven enzymes.

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\begin{array}{|l|c|c|c|c|c|}
\hline
\text{Species and strain} & \text{6PGDH} & \text{LDH} & \text{G6PDH} & \text{GDH} \\
\hline
\text{P. cystidiosus} & \text{IFO 9379} & 0.38 & 0.39 & 0.41 & 0.22 & 0.40 & 0.32 & 0.40 & 0.42 & 0.44 \\
\text{P. salmonoseomamineus} & \text{IFO 9573} & 0.52 & & & & & & & & \\
\text{P. cornucopiae} & \text{No. 1} & 0.26 & 0.28 & 0.32 & 0.37 & 0.51 & 0.28 & 0.30 & 0.32 & 0.34 & 0.51 & 0.15 & 0.30 & 0.31 & 0.33 & 0.36 & 0.47 & 0.48 & 0.59 \\
& \text{No. 2} & 0.26 & 0.28 & 0.32 & 0.34 & 0.51 & 0.28 & 0.30 & 0.32 & 0.34 & 0.51 & N. D.* & 0.15 & 0.30 & 0.33 & 0.36 & 0.47 & 0.52 & 0.59 \\
& \text{No. 3} & 0.26 & 0.28 & 0.32 & 0.37 & 0.51 & 0.28 & 0.30 & 0.32 & 0.34 & 0.51 & 0.15 & 0.30 & 0.31 & 0.33 & 0.36 & 0.59 \\
& \text{No. 4} & 0.32 & 0.37 & 0.51 & 0.28 & 0.30 & 0.32 & 0.34 & 0.51 & 0.41 & 0.54 & 0.15 & 0.30 & 0.31 & 0.33 & 0.36 & 0.59 \\
\text{P. ostreatus} & \text{KH37} & 0.44 & & & & 0.44 & & & & & 0.15 & 0.44 \\
& \text{ATCC 60691} & 0.44 & & & & 0.44 & & & & & 0.15 & 0.44 \\
\text{P. spodoleucus} & \text{No. 1} & 0.44 & & & & 0.44 & & & & & 0.15 & 0.44 \\
& \text{No. 2} & 0.44 & & & & 0.44 & & & & & 0.15 & 0.44 \\
\text{P. pulmonarius} & \text{KA01} & 0.44 & & & & 0.44 & & & & & 0.15 & 0.44 \\
\hline
\end{array}
\]

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\begin{array}{|l|c|c|c|c|}
\hline
\text{Species and strain} & \text{ADH} & \text{MDH} & \text{Est} \\
\hline
\text{P. cystidiosus} & \text{IFO 9379} & 0.31 & 0.39 & 0.42 & 0.37 & 0.40 & 0.42 & 0.47 & 0.48 & 0.50 & 0.53 & 0.65 & 0.77 & 0.80 & 0.83 \\
\text{P. salmonoseomamineus} & \text{IFO 9573} & 0.40 & 0.44 & & 0.41 & 0.44 & 0.47 & & & & 0.36 & 0.62 \\
\text{P. cornucopiae} & \text{No. 1} & 0.26 & 0.27 & 0.29 & 0.31 & 0.40 & 0.52 & 0.59 & & & & 0.62 \\
& \text{No. 2} & 0.26 & 0.27 & 0.29 & 0.31 & 0.40 & 0.52 & 0.59 & & & & 0.62 \\
& \text{No. 3} & 0.26 & 0.27 & 0.29 & 0.31 & 0.40 & 0.52 & 0.59 & & & & 0.36 & 0.62 \\
& \text{No. 4} & 0.26 & 0.27 & 0.29 & 0.31 & 0.40 & 0.52 & 0.59 & 0.26 & 0.27 & 0.19 & 0.30 & 0.36 & 0.39 & 0.36 & 0.62 \\
\text{P. ostreatus} & \text{KH37} & 0.42 & & & 0.36 & 0.39 & 0.43 & & & & 0.17 & 0.23 & 0.80 & 0.82 \\
& \text{ATCC 60691} & 0.42 & & & 0.36 & 0.39 & 0.43 & 0.50 & 0.53 & & & 0.17 & 0.23 & 0.82 \\
\text{P. spodoleucus} & \text{No. 1} & 0.42 & & & 0.33 & 0.36 & 0.39 & 0.43 & 0.49 & 0.51 & 0.52 & 0.51 & 0.17 & 0.23 & 0.54 & 0.78 & 0.80 \\
& \text{No. 2} & 0.42 & & & 0.36 & 0.39 & 0.43 & 0.49 & 0.50 & 0.51 & & & 0.05 & 0.17 & 0.22 & 0.25 & 0.78 & 0.80 \\
\text{P. pulmonarius} & \text{KA01} & 0.35 & 0.36 & 0.37 & 0.42 & 0.36 & 0.39 & 0.43 & 0.48 & 0.51 & 0.52 & 0.55 & 0.40 & 0.42 & 0.54 & 0.62 & 0.74 & 0.78 & 0.80 \\
\hline
\end{array}
\]

N. D. *: Not determined.
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Result of crossing experiment

Monokaryons belonging to different mating types were isolated from sporocarps grown in our laboratory. Four monokaryons (a, b, c, d) of *P. ostreatus* KH37, two (1, 2) of *P. spodoleucus* No. 1 and three (1, 2, 3) from *P. pulmonarius* KA01 were paired with each other (Table 4).

*P. spodoleucus* No. 1, *P. ostreatus* KH37 and *P. pulmonarius* KA01 interbred.

DISCUSSION

Although their fruiting bodies are barely distinguishable from each other morphologically, *P. spodoleucus* and *P. ostreatus* were assumed to belong to different species because of their different fructification temperatures. The temperature required by *P. spodoleucus* to form fruiting bodies lies between 8–13°C, that of *P. ostreatus* KH37 is 15–17°C. But the similarity index between these two was 88.7% (Table 3), larger than the 85.5% Sm between the isolates No. 1 and 4 of
a species *P. cornucopiae*.

Taking both *Sm* values and the compatibility into consideration, we concluded that *P. spodoleucus* could not be distinguished from *P. ostreatus* as an independent species.

Eger (4) reported that *P. pulmonarius* is a temperature-tolerant form of *P. ostreatus* with fruiting ability not blocked by temperatures of 20°C or above. But Ohira defined it as a different species from *P. ostreatus* (12, 13), distinguishable from *P. ostreatus* by fertility tests (12), but not by morphological characters. In our laboratory, when cultivated on sawdust-rice bran medium and put under identical environmental conditions, the fruiting bodies could be clearly distinguished morphologically by the pale colored, almost white pilei of *P. pulmonarius* while the *P. ostreatus* pilei were gray. This is reflected in the *Sm* (70.4%) which is smaller than that between *P. ostreatus* and *P. spodoleucus* (Table 3). But the crossing experiment (Table 4) indicates that they are very closely related to each other. Therefore, we support the view of Eger (4), and treat *P. pulmonarius* KA as a variant of *P. ostreatus*.

Still, because *P. pulmonarius* KA01 was different from Ohira’s strain, comparison should be made with other *P. pulmonarius* strains, including Ohira’s to reach a precise conclusion.

To visualize the overall relationships among the species of *Pleurotus*, a dendrogram was prepared using a single-linkage procedure (Fig. 6). Strains belonging to the species *Pleurotus cornucopiae*, *P. ostreatus* and *P. spodoleucus* formed a cluster linked at >85.5% correlation. *P. cystidiosus* and *P. salmoneostreamineus* were linked at low levels with others, which was not contradictory to the discrimination made by morphological features.
In conclusion, distinguishing species by the similarity index of the seven enzymes offered reasonable bases for classifying the *Pleurotus* species in Japan.

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REFERENCES


