電氣泳動法(Disc)에 의한 高等菌類의 몇개 種間에 있어서 蛋白質 및 酵素의 比較

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Electrophoretic Comparison of Mycelial Protein and Enzyme Patterns in Three Interspecies of Some Edible Fleshy Fungi

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ABSTRACT

Taxonomic relations among three species of some edible fleshy fungi (*Lentinus edodes*, *Pleurotus ostreatus*, *Flammulina velutipes*) in the family Tricholomataceae were examined by using polyacrylamide gel disc electrophoresis. The soluble, crude extract of mycelium grown on potato sucrose broth was subjected to electrophoresis. Similarities in the protein bands for each isolate of one species were compared with those for others. In the banding patterns there was a closer relationship between isolates within one species than among isolates of different species. However, the isozyme patterns obtained from each isolate of *Pleurotus ostreatus* (esterase, peroxidase, tyrosinase) were appeared to represent the degree of geographical variability within one species.

INTRODUCTION

The advent of recent biochemical techniques has presented useful informations about taxonomic and phylogenic relationship among the species to the fungal taxonomists who have used morphological, cytological, physiological, pathogenical, and cultural methods of classification.

In the case of need to identify a newly isolated organism or to prepare a detailed classification, the microbiologists have developed a wide range of tests to build up a biochemical profile which may reflect many aspects of the metaboism of the organism and which may be compared with similar profiles of other organisms. However, enzyme molecules are complex entities, their enzymically active sites may concern only small areas of the molecule and there may be extensive scope for variation in the rest of it. Therefore, it implies important meaning of any direct relationship at genetic level in this respect that two organisms produce exactly the same enzyme or not.

Several researchers have demonstrated the usefulness of electrophoretic analysis of proteins and various enzymes in microbial cell extract for the

determination of relationships between different taxa (Dessauer and Fox, 1964). Since Ornstein and Davis(1964) have proposed polyacrylamide gel as anticonvection medium for electrophoresis and its successful use, numbers of investigators have found that polyacrylamide gel disc electrophoresis, as a tool for taxonomic criteria of different fungi, is superior to starch gel and paper methods because of the greater number and better resolution of bands obtained.

Numerous fungi successfully identified with disc electrophoresis include *Puccinia* species (Shipton and Fleishman, 1969; Eyal *et al.*, 1967), *Puccinia* races by application of specific enzyme stains (Macko *et al.*, 1967), *Phytophthora* species (Gill and Powell, 1968a), *Colletotricum* species and *Glomerella* species (McCombs and Winstead, 1963), *Septoria* species (Durbin, 1966), *Ceratocystis* species (Stipes, 1970), two species of *Verticillium* and *Fusarium oxysporum* Fr. f. *pisi* Lindf. (Whitney *et al.*, 1968), other *Fusarium* species (Glynn and Ried, 1969; Meyer and Renard, 1969), *Candida* species and *Dermatophytes* species (Shechter *et al.*, 1966, 1968), *Penicillium* species (Bent, 1967), *Aspergillus* species (Kulik and Brooks, 1970), and *Streptomyces* species (Gottlieb and Hepden, 1966).

Since Zeldrin and Ward(1963) suggested electrophoresis in acrylamide may have taxonomic application to the slime mold, Frank and Berry(1972) reported the results of applying disc electrophoresis to some species of the order Physarales and compared pigment system with each other.

Kalab and Matloca(1966) examined mushroom species in acrylamide gel. Constantinides and Bedford(1967) also examined mushroom species for the enzyme phenoloxidase. But they did not apply their findings taxonomically. The potential of gel electrophoresis of proteins for species identification has been demonstrated in the genus *Neurospora* by Chang *et al.*(1962) and Barber *et al* (1969). Stuma and van Went(1968) have indicated its sensitivity when it was applied to *Allomyces arbuscula* Butler.

Shannon, Ballad, and Harris(1973) have determined the banding patterns in starch gel electrophoresis of some enzymes found among species of the genus *Polyporus*.

The purpose of this study is to investigate the disc electrophoretic patterns when the technique was applied to some species of Tricholomataceae classified on conventional system, in order to evaluate the usefulness of such patterns as taxonomic indicators.

MATERIALS AND METHODS

The species of Tricholomataceae used in this study, and their sources are shown in Table 1.

The isolates were maintained on 20% potato sucrose agar slants and cultures to be extracted were grown at 25°C in darkness on 500m*l* Erlenmeyer flasks containing 150m*l* potato sucrose broth media. Isolates were cultured for a period of 18 days on stationary to assure sufficient mycelial harvest for the purpose of extraction. At the end of the incubation period, the mycelial mat was recovered by filtration, washed several times with distilled water and cold buffer (0.05*N* tris-glycine, *p*H 8.3, in

Table. 1 List of isolates used in this study, their identification numbers, collection date, and source

species	Isolate no.	Date	Source
Lentinus edodes	GG 6	1963.6	Mt. Jiri
Lentinus edodes	KK 2	1962.5	Gapyeong
Lentinus edodes	W4-1	1972.4	Japan (Mori Industrial Company)
Lentinus edodes	N0103-1	1960.5	Owiljeongsa
Lentinus edodes	N0105-2	1960.5	Mt. Hanra
Lentinus edodes	16-3	1967.5	Japan (Forestry Experiment Station)
Pleurotus ostreatus	Po 2	1963.11	Anyang (Forestry Experiment Station)
Pleurotus ostreatus	Po 3	1963.8	Jeongneung (Forest By-product Center)
Pleurotus ostreatus	Po 7	1965.11	Jeongneung
Pleurotus ostreatus	Po 8	1965.11	Jeongneung
Pleurotus ostreatus	Po 9	1967.9	Japan
Pleurotus ostreatus	Po 10	1968.5	Japan
Pleurotus ostreatus	Po 12	1970.10	Jeongneung
Flammulina velutipes	Fv 3	1963.8	Jeongneung (Forest By-product Center)
Flammulina velutipes	Fv 7	1965.10	Jeongneung
Flammulina velutipes	Fv 9	1965.10	Jeongneung
Flammulina velutipes	Fv 11	1967.9	Japan
Flammulina velutipes	Fv 162	1967.5	Japan

distilled, deionized water). The mat was squeezed manually and allowed to stand in ice box at −4°C for 24 hrs.

Materials were mixed with alumina powder and then ground with a cold homogenizer. The mycelial suspension was centrifuged in International model PR-2 refrigerated centrifuge at 12,000 ×g for 60 mins. The resulting supernatant was decanted and stored in refrigerator at −20°C.

The method of disc electrophoresis of Davis was used with the following exceptions:

a. The running (lower) gel was 7.5% instead of 7.0% acrylamide. Other concentrations for the running gel were tried, ranging from 4% to 11% acrylamide, but the 7.5% acrylamide concentration gave the highest resolution and greatest number of bands.

b. In order to achieve a perfectly even surface at the lower end of the gel, 100μl of 40% sucrose solution was placed into the bottom of the glass tube.

c. The glass tube used in this studies were 5 mm in inner diameter and 80mm in length. All the tubes were filled with the running gel above the bottom of the gel tube to a depth of about 50mm and then filled with the spacer gel onto the surface of the running gel to a depth of about 10mm.

d. The sample solution (supernatant extract) was placed directly above the spacer (upper) gel with a capillary and carefully layered with the tris-glycine buffer (*p*H 8.3) immediately prior to electrophoresis.

The amount of protein per 1 m*l* of supernatant extract varied a little for the different isolates as determined by

Lowry's method (Lowry *et al.*, 1951) using human serum albumin as a standard. To prepare the sample for electrophoresis, supernatant was made to equal the protein content by adding 0.05N tris-glycine buffer. And then it was applied in 10% sucrose solution for retaining enzyme activity.

To study general protein and other enzymes, 0.2ml of sample (containing 500μg) layered on the upper gel produced most satisfactory results. Electrophoresis was carried out on a custom-built electrophoretic apparatus in a cold room at 4°C. The sample passed through the upper gel at 2mA per tube and at 4mA per tube in the lower gel until the tracking dye (bromophenol blue) front reached a point 45mm from the surface of the running gel. To avoid heat denaturation for enzyme, it is useful to reduce the current to 3mA per tube in the lower gel.

After electrophoresis, gels were removed and stained immediately. General protein was stained for 60 mins in 0.1% naphthol blue black in 7% acetic acid and destained in 7% acetic acid for 48 hrs. Esterase activity was detected by the procedure of Hunter and Burstone (1960) with 1% α-naphthyl acetate as the substrate and fast blue RR salt as the dye coupler. Peroxidase activity was assayed by the benzidine method of Scandalio(1965). Tyrosinase activity was stained by the method of Jalley and Mason(1969).

All gels were examined on fluorescent lamps and diagramed immediately after fixing the gel.

RESULTS AND DISCUSSION

Electrophoretic patterns produced with general protein, esterase, peroxidase, tyrosinase stains are shown in Figs. 1-6.

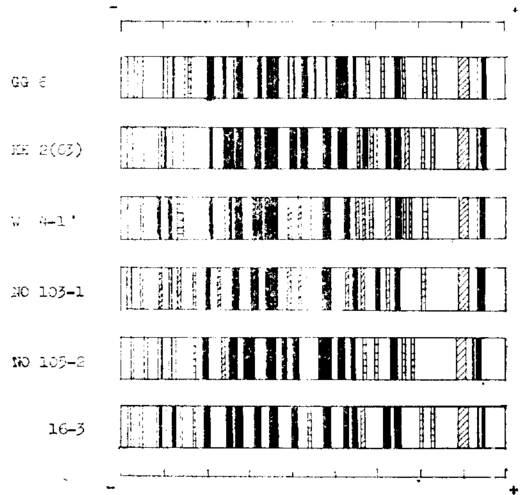

Fig. 1. The disc electrophoretic separation on polyacrylamide gel of soluble proteins from 6 isolates of *Lentinus edodes*. Black bands and lines represent the densest protein deposits, barred bands the least dense, and cross-hatched and dotted lines those of intermediate density.

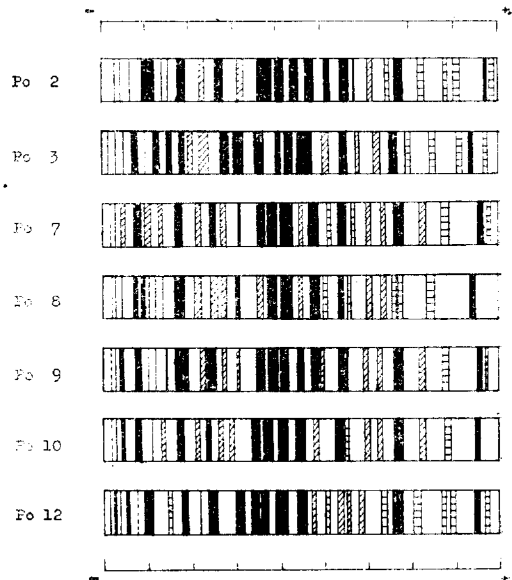

Fig. 2. The disc electrophoretic separation on polyacrylamide gel of soluble proteins from 7 isolates of *Pleurotus ostreatus*. Black bands and lines represent the densest protein deposits, barred bands the least dense, and cross-hatched and dotted lines those of intermediate density.

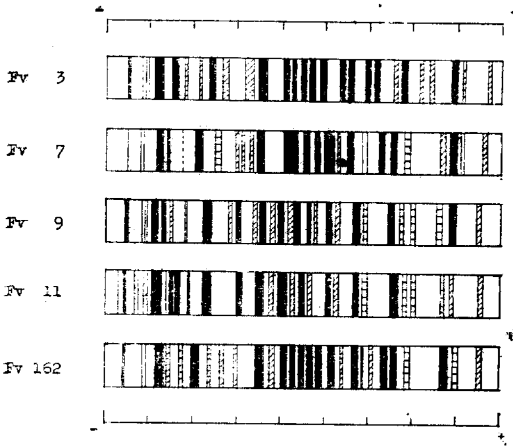

Fig. 3. The disc electrophoretic separation on polyacrylamide gel of soluble proteins from 5 isolates of *Flammulina velutipes*. Black bands and lines represent the densest protein deposits, barred bands the least dense, and crosshatched and dotted lines those of intermediate densiy.

Polyacrylamide gel electrophoretic patterns of the soluble proteins from the 18 isolates revealed a maximum of 31 protein fractions and a minimum of 28 protein fractions from *Lentinus edodes*, a maximum of 27 and a minimum of 24 protein fractions from *Pleurotus ostreatus*, and a minimum of 27 protein factions from *Flammulina velutipes*.

Investigators who have studied proteins of other fungi with polyacrylamide gel electrophoresis reported generally fewer protein fractions. Chang *et al.* (1962) resolved 25 bands of *Neurospora* spp. Shecher *et al.* (1966) obtained a maximum of 15 protein fractions from preparations of dermatophytic fungi. Durbin (1966) reported 13 bands from 3 *Septoria* spp. Gill and Powell (1968) obtained from 10 to 13 "components" from 3 spp. of *Phytophthora*. Stipes (1970) reported a minimum of 10 and a maxi-

Table 2 Mycelial protein fractions on polyacrylamide gels of three species in Tricholomataceae

Species	Isolate no.	No. bands	No. bands common to isolates of same species
Lentinus edodes	GG 6	31	10
Lentinus edodes	KK 2	31	
Lentinus edodes	W 4—1	31	
Lentinus edodes	NO103—1	29	
Lentinus edodes	NO105—2	28	
Lentinus edodes	16—3	30	
Pleurotus ostreatus	Po 2	25	8
Pleurotus ostreatus	Po 3	26	
Pleurotus ostreatus	Po 7	26	
Pleurotus ostreatus	Po 8	27	
Pleurotus ostreatus	Po 9	26	
Pleurotus ostreatus	Po 10	24	
Pleurotus ostreatus	Po 12	27	
Flammulina velutipes	Fv 3	28	10
Flammulina velutipes	Fv 7	29	
Flammulina velutipes	Fv 9	29	
Flammulina velutipes	Fv 11	27	
Flammulina velutipes	Fv 162	28	

mum of 15 separate proteins from 4 spp. of *Ceratocystis*. Recently, Kulik and Brooks obtained a maximum of 48 and a minimum of 29 protein fractions from 3 strains of each of 7 *Aspergillus* spp.

The number of separated proteins of the 18 isolates obtained from diverse geographical areas and the number of common protein fractions among the same species are shown in Table 2.

To assure that these characteristic banding patterns were consistantly reproducible, multiple samples from at least four cultures of each isolate were subjected to electrophoresis. In fact, many workers agree that differences in age of culture, type of growth medium, and growth temperature are possible sources of misinterpretation and can only be eliminated by careful maintenance of cultural condition.

The migration distance of all general protein bands from each isolate were compared with those of the bands from every other isolate. The wide bands of some enzymes appeared to represent heterogeneous molecular moieties which were not separable under the conditions of this experiment. All 18 isolates have one common protein fraction at 20 mm. The 6 isolates of *Lentinus edodes* and the 7 isolates of *Pleurotus ostreatus* have 4 common protein fractions at 1.5, 18, 20, and 24mm. The 7 isolates of *Pleurotus ostreatus* and the 5 isolates of *Flammulina velutipes* have 2 common protein fractions at 20 and 30.3mm. The isolates of *Lentinus edodes* and of *Flammulina velutipes* have 3 common protein fractions at 6, 20 and 40mm.

Upon examination of the results from the profile of protein bands with the

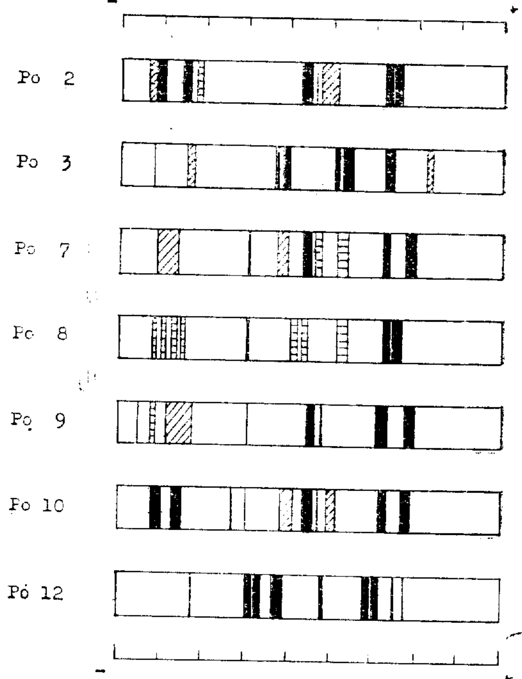

Fig. 4. Isozyme patterns of esterase from 7 isolates of *Pleurotus ostreatus* produced with disc electrophoresis in polyacrylamide. Black bands and lines represent the densest enzyme deposits, barred bands the least dense, and cross-hatched bands those of intermediate density.

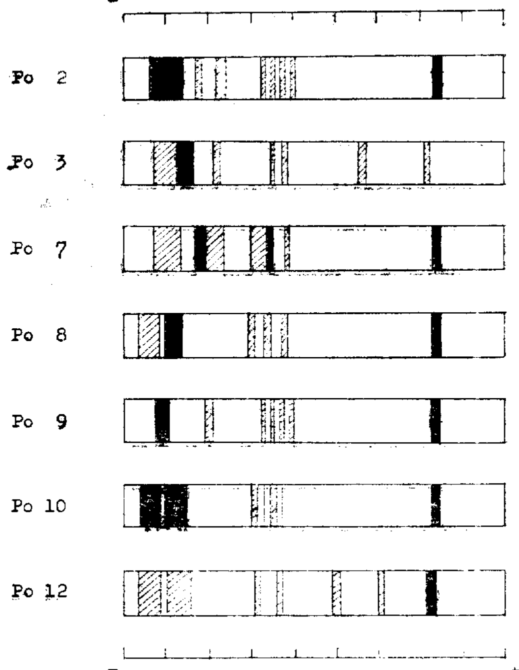

Fig. 5. Isozyme patterns of peroxidase from 7 isolates of *Pleurotus ostreatus* produced with disc electrophoresis in polyacrylamide. Black bands and lines represent denser enzyme deposits than cross-hatched bands.

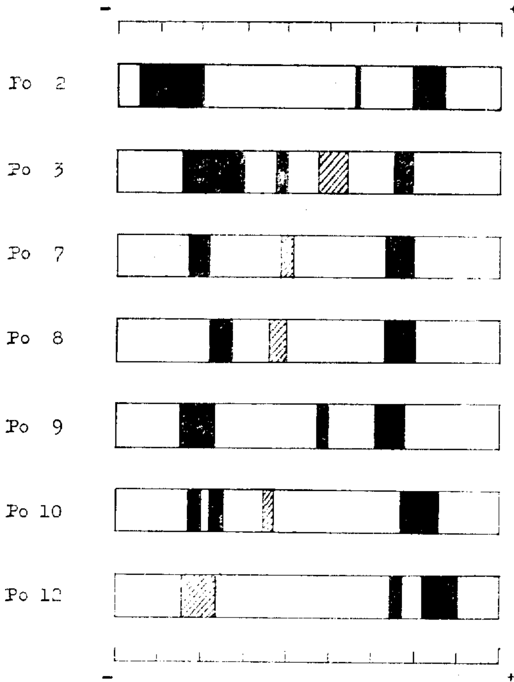

Fig. 6. Isozyme patterns of tyrosinase from 7 isolates of *Pleurotus ostreatus* produced with disc electrophoresis in polyacrylamide. Black bands represent denser enzyme deposits than cross-hatched bands.

migration distance, with only a few exceptions, it may be concluded that isolates of the same species show greater similarities in banding patterns than are found when the isolates of different species are compared. However, when the isozyme patterns for a given enzyme of each isolate of a single species (*Pleurotus ostreatus*) are compared, they are observed to be unique to that indivisual isolate. This isozyme pattern was a great help to determine the degree of variability which may occur within one species.

Therefore, these results indicate that the use of electrophoretic banding patterns of the soluble protein for identifying some species of Tricholomataceae could present a valuable information to conventional taxonomic techniques. Moreover, it should be cautioned that there are variants within *Pleurotus ostreatus* species which differ in isozyme patterns as they do in morphology. The results of this investigation also illustrate the fact that it is possible to demonstrate differences between the species and within the species by electrophoretic techniques. However, since there may be many geographic isolates of morphologically determined species, in order to utilize the results of elecrophoresis to establish taxonomic relationships, more enzymes, more species and more isolates must be studied.

摘 要

Tricholomataceae科에 屬하는 食用菌類인 *Lentinus edodes, Pleurotus ostreatus, Flammulina velutipes*의 세 種間에서 分類學的인 關係를 考察하기 위하여 disc 電氣泳動法을 利用한 硏究를 試圖하였다.

감자 배지 상에서 기른 이들 菌類의 菌糸로 부터 추출한 水溶性 抽出物을 電氣泳動에 使用하였다. 이들 각 菌株에 대한 蛋白質 分離에서 이들 相互間의 類以性을 다른種의 菌株와 비교한 結果 한 種間에서 더욱 가까운 關聯을 볼 수 있었다.

그러나 *Pleurotus ostreatus*의 각 菌株로 부터 얻어진 esterase, peroxidase, tyrosinase의 isozyme pattern은 한 種間에서의 地域的인 變異性의 程度를 보여 주는것으로 생각된다.

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