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Electrophoretic Patterns of Isozymes from the Mycelia of the Auxotrophs of *Lentinula edodes*

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표고버섯 영양요구성 변이주의 전기영동법에 의한 Isozyme 비교

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Abstract: The Isozyme activities of *Lentinula edodes* were studied as a preliminary study for genetic analysis after protoplast fusion. The presence of peroxidase, esterase, superoxide dismutase, acid phosphatase, alkaline phosphatase, alcohol dehydrogenase and α-amylase was examined. An intracellular buffer-soluble protein from the mycelia was used for enzyme analysis on nondenaturing polyacrylamide gels. The auxotrophs of *Lentinula edodes* were positive for peroxidase, esterase, superoxide dismutase and acid phosphatase. However, alkaline phosphatase, alcohol dehydrogenase and α-amylase were not detected. The esterase and peroxidase were not affected by the various culture age. Isozyme identification may be a useful tool after protoplast fusion.

KEYWORDS: Auxotroph, Electrophoresis, Isozyme, *Lentinula edodes*, Nondenaturing gel

Isozyme markers have been used for genotypic and genetic analysis in several fungi, and descriptions of general techniques and of numerous enzyme assay systems have been published (Berry and Franke, 1973; Kerrigan and Ross, 1989; Park *et al.*, 1988; Roux and Labarere, 1990; Royse and May 1981; Szklarz *et al.*, 1989). By analysing enzyme activities with high polymorphism and variability, it is possible to characterize strains.

It has been shown that peroxidase participates in lignin decomposition in cooperation with other fungal enzymes (Lobarzewski and Paszczynski, 1985; Szklarz and Leonowicz, 1986). Peroxidase activities were studied in *Fusarium* species, *Coriolus versicolor, Phellinus igniarius, Lycoperdon* sp. (Sato *et al.*, 1980; Harkin and Obst, 1973; Szklarz *et al.*, 1989), *Agaricus bisporus* (Paranjpe *et al.*, 1979), and *Pleurotus* spp. (Park *et al.*, 1988).

Esterase activity was detected in *Agaricus bistorquis* (Roux and Labarere, 1990), *Lentinula edodes* (Ohmasa and Furukawa, 1986), *Pleurotus* spp. (Park *et al.*, 1988) and *Ganoderma* species (Park *et al.*, 1989).

Superoxide dismutases are present in all aerobic organisms. They play a significant role in protecting the fungal cells from damage by air pollutants. They belong to the family of metalloenzymes catalyzing the disproportionation of superoxide radicals (O_2^-) to yield molecular oxygen and H_2O_2.

Acid phosphatase was determined in *A. bistorquis* (Roux and Labarere, 1990), and the cellular content compared to alkaline phosphatase was reported by Leatham (1984).

In this study, we examined several electrophoretic isozyme patterns from the my-

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celia of auxotrophs of *L. edodes* and also examined the effects of mycelial culture age on the peroxidase and esterase activity.

Materials and Methods

Reagents

Growth supplements were purchased from Difco Laboratories (Detroit, MI, USA). Reagents were obtained from Sigma Chem. Co. (St. Louis, MO, USA), if not mentioned.

Strains and culture condition

Ultraviolet ray induced auxotrophs, LE207 (Ser^-) and LE(eb)26 (Ile^-, Arg^-, Thy^-) of *L. edodes* (Berk.) were used (Kim *et al.*, 1996). Media consist of glucose 20 g, yeast extract 2.0 g, peptone 2.0 g, K_2HPO_4 1.0 g, KH_2PO_4 0.46 g, $MgSO_4 \cdot 7H_2O$ 0.5 g per liter. Mycelia were inoculated in 15 m*l* of liquid media, and were stationary incubated at 30°C in the dark.

Cell lysates and electrophoresis

After the culture was grown for a certain time, specified under the results, the mycelium was collected and rinsed with ice cold 0.1 M Tris-HCl (pH 7.6). The mycelium was homogenized with dry ice and centrifugated at 15000 rpm for 50 min at 4°C. The supernatant retaining enzymes were quantitated by Lowry method with BSA as a standard. Enzyme activities were determined after electrophoretic migration on 5~10% nondenaturing polyacrylamide gels. The methods rely on the production of a colored final product. The presence of intracellular peroxidase, esterase, superoxide dismutase, acid phosphatase, alkaline phosphatase, alcohol dehydrogenase and α-amylase were examined on the nondenaturing polyacrylamide gels.

Determination of enzyme activities

Peroxidase (EC 1.11.1.7) Peroxidase was detected by a modified method of Tanksley (1979). Briefly, gels were incubated in TMBZ solution (3,3',5,5'-tetramethyl benzidine 25 mg, methanol 25 m*l*, 1 M Na-acetate 50 m*l*, pH 4.7) for 30 min at 30°C in the dark, and then reacted with 1 m*l* of 3% H_2O_2. The TMBZ is a substrate of peroxidase activities and is oxidized by the hydrogen peroxide to give a black product.

Superoxide dismutase (EC 1.15.1.1) Staining for superoxide dismutase activity in gels was performed as described by Beauchamp and Fridovich (1971). The gels incubated in a solution containing 5 mg tetrazolium bromide (MTT) and 5 mg phenazine methosulfate (PMS) in 50 m*l* 0.05 M Tris-HCl, pH 7.5, for 20 min at 30°C in the dark, and transferred, incubated in solution containing 1 mg riboflavin and 0.2 m*l* TEMED (N,N,N',N'-tetramethylethylenediamine) in 50 m*l* of 0.05 M Tris-HCl, pH 7.5. After incubation, gels were exposed to the light for 15 min.

Esterase (EC 3.1) The gels were fixed in citrate-acetone-methanol fixative (38.3 mM citrate 18 m*l*, acetone 27 m*l*, methanol 5 m*l*) for 30 seconds, washed twice with deionized water, and air-dried for 20 min at room temperature. The dried gels were incubated for 30 min at 37°C in the solution mixture: 10 mg of Fast Garnert GBC salt in 50 m*l* of 20 mM tris-HCl buffer, pH 7.6; 20 mg of α-naphthyl acetate in 2 m*l* of ethylene glycol monomethyl ether. The naphtol radical of the carboxylic esters is released by esterase and joins with diazonium salt to form an insoluble black product.

Acid phosphatase (EC 3.1.3.2) Acid phosphatase was detected by a modified method of Scandalios (1969). The gels incubated in acetate buffer (pH 5.0) for 30 min at 5°C, and in the solution mixture: 1 M $MgCl_2 \cdot 6H_2O$ 0.5 m*l* and 50 mg of Fast Garnert GBC salt in 50 m*l* of 50 mM Na-acetate buffer; 1.5 m*l* of 1% β-naphthyl acid phosphate dissolved in 50%

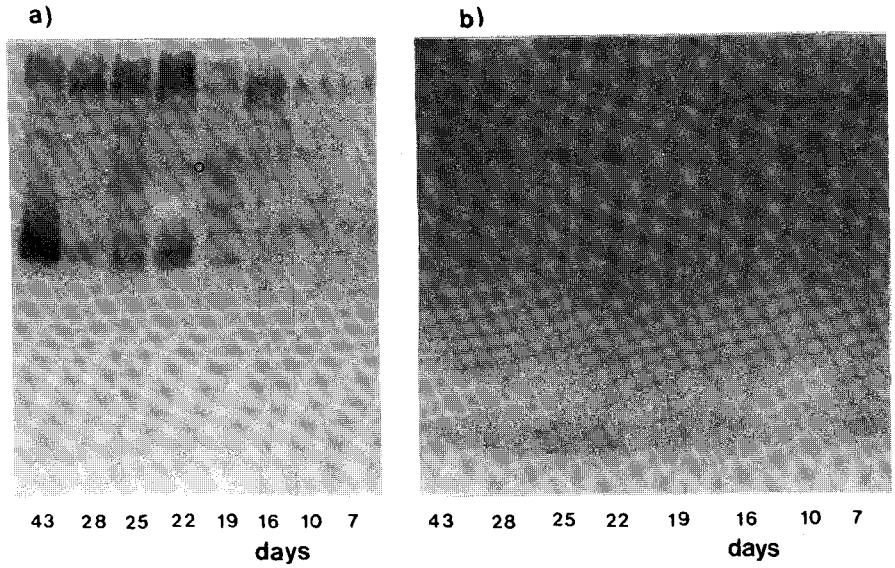

Fig. 1. Changes of electrophoretic isozyme patterns of the serine requring auxotrophs of *L. edodes*, LE 207, with culture period; (a) peroxidase, (b) esterase.

acetone, for 1~5 hrs at 30°C.

Alcohol dehydrogenase (EC 1.1.1.1) Alcohol dehydrogenase staining was performed by a modification of Vallejos (1983). The gels were incubated for 15~60 min at 30°C in the dark in the detection mixture: 0.05 M Tris-HCl, pH 8.6; nicotinamide adenine dinucleotide (NAD) 40 mg; phenazine methosulfate (PMS) 20 mg; nitroblue tetrazolium (NBT) 20 mg; 6 m*l* of ethanol. The oxidation of ethanol by alcohol dehydrogenase coupled to NAD allows, after two intermediate steps, reduction of tetrazolium salt to insoluble dark blue formazan.

α-Amylase (EC 3.2.1.1) The gels were incubated in 0.05 M phosphate buffer (pH 6.9) containing soluble starch for 30 min. After washed with distilled water, they were reacted with iodine solution.

Results

Time course of peroxidase and esterase

The effect of culture period on peroxidase and esterase isozyme patterns was examined for LE207. Two peroxidase bands were detected by the reaction with TMBZ and H_2O_2. The low molecular weight band appeared after 19-day cultures and stable through cell culture period. The esterase band began to appear after four days of culture. Although the staining intensity of the bands varied from 7-day to 43-day cultures, the band pattern did not change (Fig. 1). The LE(eb)26 was stable through cell culture period (data not shown).

Enzyme activities

Electrophoretic comparison of the two auxotrophs of *L. edodes* on a nondenaturing gel stained for the peroxidase activity showed that two strains had different isozymes. LE 207 showed two activities that had distinctly different mobilities, but LE(eb)26 had a broad band.

The esterase patterns of LE207 and Le(eb) 26 were similar. Optima for pH on esterase activity of LE207 was detected at 620 nm by reacting 100 μ*l* of sample with 2 m*l* of Fast Garnert GBC salt (200 μg/m*l*) and 2 m*l* of α-

naphthyl acetate (400 μg/ml) at 37°C. Maximum esterase activity was detected at pH 7.5 (Table 1).

Two transluminant superoxide dismutase bands and two acid phosphatase bands were detected (Fig. 2). Any alkaline phosphatase was not detected when incubated by diazonium in naphthyl solution. Alcohol dehydrogenase and α-amylase activities were not detected by the method which was used in this study.

Discussion

Isozyme analysis by gel electrophoresis is a powerful method for taxonomic, genetic and population studies. It seems more reliable than morphological description, and easier and faster than restriction fragment length polymorphism analysis.

L. edodes possessed a high variability in isozyme activities. In *L. edodes* one to four bands for peroxidase were found. Ohmasa and Furukawa (1986) reported a total of 37 isozymes of esterase. And a total of 16 esterase isozyme bands for 24 strains among 45 isolates was reported in *L. edodes* by Toyomasu and Zennyozi (1981). The intracellular content of acid phosphatase was lower than alkaline phosphatase but the enzyme activity was about 100 times higher than that of alkaline phosphatase in *L. edodes* (Leatham, 1984). Strong acid phosphatase band activity was detected, whereas no intracellular alkaline phosphatase activity was detected in this experiment. The protein contents of the sample for the isozyme assay were detected

Table 1. Effects of pH on the esterase activity of *Lentinula edodes*

Strain	pH		
	4.5	6.5	7.5
LE207	0.55[a]	0.45	1.45

[a]Absorbance at 620 nm

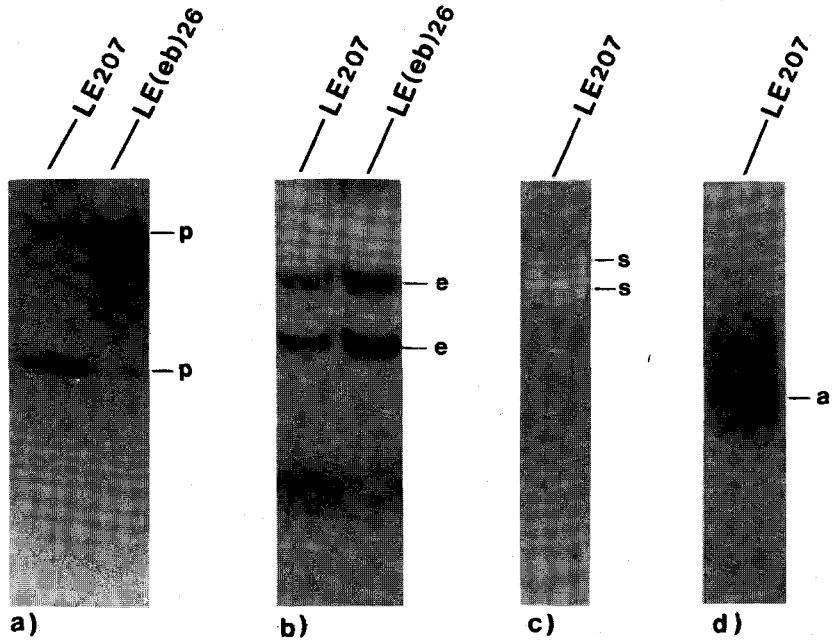

Fig. 2. Activity staining of peroxidase (a), esterase (b), superoxide dismutase (c) and acid phosphatase (d) of auxotrophs of *L. edodes* on nondenaturing gels after 25-day cultures. Abbreviations for isozyme bands are p, peroxidase; e, esterase; s, superoxide dismutase; a, acid phosphatase.

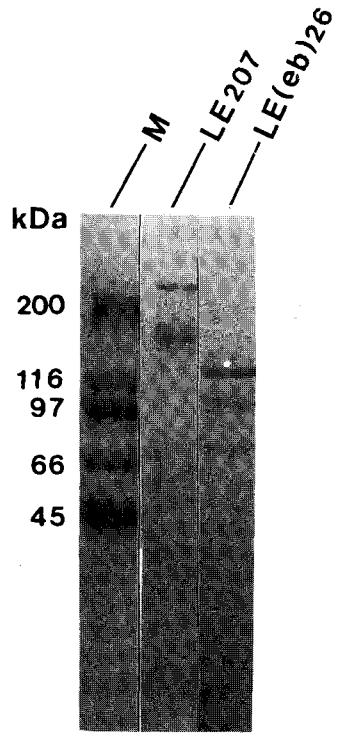

Fig. 3. SDS-PAGE seperation of the water soluble total proteins from the mycela of LE 207 and LE(eb)26 after 25-day cultures, auxotrophs of *L. edodes*. Gel was stained with Coomassie Brilliant Blue after electrophoresis.

by the Coomassie Brilliant Blue staining after SDS-PAGE (Fig. 3).

In general, the culture age and the composition of the media are very important, because isozyme patterns change with those conditions. In *L. edodes*, the esterase patterns at different culture ages did not change, whereas media composition changed isozyme patterns significantly (Ohmasa and Furukawa, 1986). In this study the peroxidase and esterase isozymes of LE207 were stable through the culture period (43 days). The effect of pH on enzyme stability is important. To determine its effect, we changed the pH of enzyme extraction buffer. Ohmasa and Furukawa (1986) reported that the isoelectric point of the esterase of *L. edodes* was present in the acidic region (pH 3.3~6.5). In this study the maximum esterase activity of LE 207 was detected at pH 7.5.

In this study, we examined some isozyme activity of *L. edodes*. The cell-free extract of *Lentinula edodes* was positive on peroxidase, esterase, superoxide dismutase and acid phosphatase. However, alkaline phosphatase, alcohol dehydrogenase and α-amylase were not detected by the method which was used in this study. By compiling these isozyme patterns, it may be possible to identify and discriminate parental strains of *L. edodes* and new hybrids of *L. edodes* with other fungi.

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적 요

표고버섯(*Lentinula edodes*)의 동위효소 양상을 표고 연구의 기초 연구의 일환으로 실시하였다. 균사체의 Tris-HCl 완충액에 용해되는 세포내 효소를 nondenaturing polyacrylamide gel 전기영동법으로 분리후, peroxidase, esterase, superoxide dismutase, acid phosphatase, alkaline phosphatase, alcohol dehydrogenase, α-amylase 효소 활성을 측정하였다. 표고는 peroxidase, esterase, superoxide dismutase, acid phosphatase 활성이 검출되었으며, 본 연구에서 사용한 방법으로 alkaline phosphatase, alcohol dehydrogenase, α-amylase 효소 활성은 검출되지 않았다. 표고버섯의 peroxidase, esterase band는 배양 기간에 따라 큰 차이가 없이 안정하였으며, 동위효소는 표고의 유전적 연구 및 원형질체 융합후 융합체의 특성 연구에 중요하다.

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