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Localization of Phenoloxidases in *Coprinus congregatus* Grown on a Low-Temperature-Liquifying Medium

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저온 액화성 응고제를 사용한 고체배지에서 자란 *Coprinus congregatus*의 phenoloxidase들의 localization

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ABSTRACT: The hyphal tip phenoloxidases of *Coprinus congregatus* were localized by the protoplast-concanavalin A method. Protoplast were generated from cultures grown on a solid medium which was solidified with a new gelling agent, Pluronic Polyol F127, instead of agar. The enzymes were associated with the cell membrane which might work as a transducer in the light recepter complex.

KEY WORDS □ ***Coprinus congregatus*; low-temperature-liquifying medium; protoplast isolation; phenoloxidases**

Coprinus congregatus Fries, one of mushroom-forming fungi, has a unifactoral multiple allele mating type (a1 to a9) (Ross, 1982a). Any homokaryotic hypha(N) has an ability to mate with any other homokaryon which has different mating type. Nuclear fusion does not occur until the fungus forms the basidium. Only the dikaryotic mycelium (N + N) can normally undergo differentiation into primordium and mushroom. When it grows in the dark, it does not show any development until it receives light (Durand & Jacques, 1982; Ross, 1982b). In dark-grown cultures, only the new growth zone (hyphal tip zone) has the competence to respond to light and also has the highest phenoloxidase activities. When a hyphal tip zone is illuminated for 3 hours, many primordia will be formed only at the illuminated zone (Ross, 1982b, 1985). The enzymes are not secreted into the agar or liquid medium (Ross, 1982c; Choi *et al.*, 1987). This allows to propose a model of the light receptor complex which places the light receptor in the cell membrane as usual (Senger, 1980), and also propose that the enzymes are associated with the cell membrane (Ross, 1985). When it grows in a liquid medium it forms sclerotia not primordia and the mycelial pellet of the liquid culture has different isozyme patterns compared with those of the hyphal tip enzymes of the agar culture by the PAGE analysis (Choi *et al.*, 1987). Therefore, the cell membranes isolated from the liquid shake culture can not be used to examine the localization of hyphal tip enzymes which are considered to be involved in the photomorphogenesis.

Pluronic Polyol F127, a block copolymer of polypropylene oxide and ethylene oxide which forms a stable gel at temperatures above 10 C and the gels liquify as the temperature drops below the critical value for the concentration used (Gardener & Jones, 1984), successfully replaces the agar as a new solidifying agent-the isozyme patterns of polyol and agar cultures on the PAGE are identical (Choi & Ross, 1988). It is therefore possible to isolate the mycelial mat grown on the polyol medium by lowering the temperature.

When any kind of biological membranes are to be isolated, it is very helpful if a membrane has a marker molecule like the chlorophyll in the chloroplast mem-

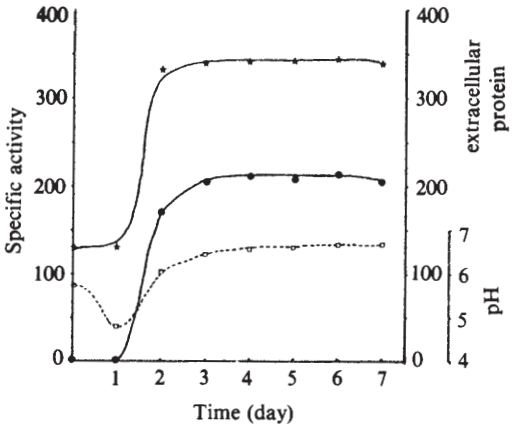

Fig. 1. *Glucanase production by T. viride.*
● —● ; specific activity (u/mg protein)
★ —★ ; extracellular protein (ug/m*l*)
□ ---- □ ; pH

brane. Since cell membrane of *C. congregatus* has no marker, however, protoplast generation is necessary for the isolation of cell membranes. *Trichoderma viride* CBS 354.33 obtained from Dr. Wessels was used for the preparation of hydrolytic enzyme which degraded coprinus cell walls (glucanase). Highly active glucanase was produced by following the methods of De. Vries & Wessels (1972), and the coprinus cell wall was used as the sole carbon source. Glucanase activity was determined by incubation of 1 m*l* of 0.5% coprinus cell wall suspension with 1 m*l* of enzyme solution at 30 C for 1 hr. The concentration of reducing sugar was determined by the Bernfeld's method (1951). One unit represented the ability to generate reducing sugar equivalent to 1 μg of glucose for 1 min. As described in Fig. 1 glucanase production reached maximum at day 4.

The dikaryon (a1 × a2) of *C. congregatus* Fries was grown on Difco Emerson's YpSs medium (Ross, 1982a) with the polyol (25%; Choi & Ross, 1988) instead of agar in 100 mm diameter plastic Petri dish at 25 °C. The hyphal tips of 4 day old culture were harvested and incubated at 4 °C for 1 hr. The liquified mycelial suspension was washed several times with water, then with 0.5 M $MgSO_4$ in 0.05 M sodium maleate buffer (pH 5.8) before finally being suspended in the same solution. Glucanase was added to the mycelial suspension (0.2%) and the mixture was incubated for 3 hours at 30 °C on a rotary shaker at 100 rpm. We usually got 1-5 × 10^8 protoplasts per 40 plates. There was usually contamination of hyphal fragments during the isolation of protoplasts by filtration method but it could be avoided by the step-gradient centrifugation method (Tilburn *et al.*, 1983). The final $MgSO_4$ concentration was increased to 1.2 M, then protoplasts were separated from mycelial debris by centrifugation at 4000 × g for 15 min with an overlay of an equal volume of 0.6 M sorbitol, 0.1 M Tris, pH 7.0. The protoplasts formed a sharp band at the interface, and they were washed twice with 0.6 M sorbitol/0.05 M HEPES buffer (pH 6.8)/5 mM $MgCl_2$ (Vermeulen *et al.*, 1979).

The protoplast suspension was mixed with an equal volume of concanavalin A (con A) solution (0.5 mg/m*l*) and then incubated at room temperature for 15 min. The mixture was centrifuged at 500 × g for 5 min and the pellet was washed with sorbitol/HEPES/$MgCl_2$ buffer. Protoplasts were lysed by suspending in 0.05 M HEPES buffer for 30 min, the suspension was overlayed on three volumes of sorbitol/HEPES/EDTA (1 mM) buffer and then centrifuged at 700 × g for 20 min (Vermeulen *et al.*, 1979). The pellet was washed with HEPES/EDTA buffer and suspended in 0.1 M α-methyl-mannoside, 0.05 M HEPES to remove con A (Perlmann *et al.*, 1970). Cell membrane fractions were cellected by centrifugation of the final suspension at 10,000 × g.

Quantitative laccase determination was performed by using o-tolidine as the enzyme substrate (Ross, 1982c). Tyrosinase activity was determined by modified method of Leonard (1971). Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

Both laccase and tyrosinase activities at each isolation step were described in Table 1. When con A was added to the protoplasts, specific activities of both enzymes were dropped by 35% to 50%. There might be two explanations for this. Con A was a lectin protein which bound with any carbohydrate moieties especially with methylmannoside (Perlmann *et al.*, 1970). Increase of a protein concentration by adding con A could cause the decrease of the specific activities. Con A was also thought to bind with the enzymes to decrease their activities-many membrane associated proteins have carbohydrate moieties. However the enzyme activities in cell membrane were recovered by 72% (tyrosinase) and 78% (laccase) after the membrane isolation. This results meant that the enzymes were associated with the cell membrane. This could be a good clue to isolate the enzymes by using a con A-sephadex column.

Even though the isozyme patterns of the liquid shake culture were quite different from those of the agar culture (Choi *et al.*, 1987), the enzymes of the former also co-banded with the membrane fractions

Table 1. *Phenoloxidase activities at every step during cell membrane isolation (mean value of three replicate samples ± standard deviation).*

enzymes	laccase			tyrosinase		
isolation steps	specific activity	total activity	yield (%)	specific activity	total activity	yield (%)
protoplasts	6.36 ± 0.12	114.48 ± 2.16	100.00	4.80 ± 0.15	86.40 ± 2.70	100.00
con A-protoplasts	3.16 ± 0.26	41.08 ± 3.38	35.88	3.16 ± 0.26	41.08 ± 3.38	47.55
osmolyzed protoplasts	4.86 ± 0.44	55.89 ± 5.06	48.82	4.05 ± 0.39	46.58 ± 4.49	53.91
cell membranes	9.87 ± 0.27	88.83 ± 2.43	77.59	6.91 ± 0.39	62.19 ± 3.51	71.98

(Ross, 1985). Since the hyphal tip enzymes had been implicated in the transduction of light signal (Ross, 1982c, 1985; Choi & Ross, 1988) and since it was almost impossible to isolate the mycelial mats from a agar culture without heat or other methods traumatic to fungal cells, a new solidifying agent which could replace agar must be used. Since polyol culture had the same isozyme patterns as the agar culture (Choi & Ross, 1988) it provided good proof for the enzyme localization. This experiment propose that phenoloxidases may be a transducer (Ross, 1985) in the light receptor complex in *C. congregatus*.

적 요

새로운 고체배지 응고제인 Pluronic Polyol F127을 한천대신 사용하여 *C. congregatus*를 배양하였고 균사 끝(hyphal tip)에 있는 phenoloxidase들의 세포내에서의 존재위치를 확인하였다. 고체배지의 균사에서 원형질체를 얻었고 concanavalin A를 처리하여 세포막을 분리하였다. 세포막 분획에 phenoloxidase들이 존재하였고 이는 이 효소가 light receptor complex의 한 부분임을 시사한다.

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