Effect of staurosporine on growth and hyphal morphology of *Pleurotus ostreatus*

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Staurosporine is a strong inhibitor of protein kinases. At low concentrations, this antibiotic inhibited the growth of both protoplasts and mycelium of *Pleurotus ostreatus*. Its minimum inhibitory concentrations were 6.5 μM and 9.6 μM for protoplasts and mycelium, respectively. At a lower concentration (0.54 μM), it caused swelling of hyphal tips and subapical regions while some hyphae became stubby and bulbous. The phenomena were compared with the morphological effects of polyoxin D, which is a competitive inhibitor of chitin synthase. The results suggest the involvement of protein kinases(s) in regulation of apical growth of fungi.

Introduction

Staurosporine was first isolated as a new alkaloid produced by *Streptomyces* sp. AM-2282 with antifungal activity (Omura *et al.*, 1977). It is also a potent inhibitor of Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C: PKC) (Tamaoki *et al.*, 1986). Further work indicated that staurosporine inhibits other serine/threonine protein kinases and also protein tyrosine kinases (Nakano *et al.*, 1987; Yamaguchi & Kathuria, 1988). Currently, it is widely used as a pharmacological tool in mammalian cells (Pardee *et al.*, 1992). In the first report (Omura *et al.*, 1977), the antifungal effects of staurosporine on *Candida* sp., *Saccharomyces* sp. and *Aspergillus* sp. were studied. However, its effect on basidiomycetes is not known.

PKC is known to regulate growth, differentiation and morphogenesis in animal cells (Nishizuka, 1987; Fargo & Nishizuka, 1990). Although nothing is yet known about PKC in the morphogenesis of fungi, PKC activity has been detected in *Neurospora crassa* (Favre & Turian, 1987) and purified from *Saccharomyces cerevisiae* (Ogita *et al.*, 1990). PKC with features similar to those of mammalian PKC was recently isolated from *Pleurotus ostreatus* (Magae, 1993).

In this work, we have studied effects of the most potent known inhibitor of protein kinases, staurosporine, on the

growth and hyphal morphology of *Pleurotus ostreatus* as a first step towards analysing the roles of protein kinase(s) in this fungus.

Methods

Chemicals. Staurosporine was isolated from the mycelium of *Actinomyces* sp., which was found in the course of our screening for PKC modulators (Osada *et al.*, 1988). Before use, its purity was checked by HPLC, on which it gave a single peak, either alone or with the authentic sample. Stock solution (1 mg ml⁻¹) was prepared in methanol and stored at -20 °C.

Polyoxin D from *Streptomyces cacaoi* and chitinase used for protoplast isolation were purchased from Calbiochem. H-7 was purchased from Seikagaku Kogyo. Cycloheximide and griseofulvin were from Sigma. Novozym 234 was from Novo Industry.

Organism and media. A dikaryotic strain, *Pleurotus ostreatus* K01, which had been formally designated *P. pulmonarius* K01, was from Kawamura Edible Mushroom Laboratory. This strain is able to differentiate basidiocarps at temperatures above 18 °C and is sexually compatible with *P. ostreatus* ATCC 60691 (Magae *et al.*, 1990). A homokaryon (K01-M2) used in this study was isolated as a single spore from strain K01. Both protoplasts and mycelium of K01-M2 were used to evaluate the effects of the antibiotics on the fungus.

Measurement of growth. K01-M2 was grown on MA (malt extract, 2%, w/v; agar, 1.8%, w/v) in a 90 mm Petri dish. When the colony had reached the edge of the Petri dish, mycelium about 1 cm inside the periphery of the colony was cut out with a cork borer (3 mm diameter) and transferred to MA in 35 mm plates containing various concentrations of staurosporine. The growth and diameter of the colony at 25 °C were determined in duplicate each day for two weeks.

Protoplasts and regeneration. Mycelium was grown in 20–30 ml of GMY (glucose, 1%, w/v; malt extract, 1%, w/v; yeast extract, 0.4%) at 24 °C for a week. The mycelium was then harvested by filtration and incubated in a 2 ml mixture of 2% (w/v) Novozym 234 and 1% (w/v) chitinase in phosphate/mannitol buffer (50 mM-phosphate buffer

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Abbreviations: PKC, Ca^{2+} /phospholipid-dependent protein kinase; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; MIC, minimum inhibitory concentration.

containing 0.6 M-mannitol, pH 5.6; PMB) for 1 h at 30 °C. After undigested mycelial debris was removed by filtration through a glass filter, protoplasts were collected by centrifugation and washed with PMB. The purified protoplasts were finally diluted to $5 \times 10^6 \text{ ml}^{-1}$ with PMB, and 0.1 ml was plated out in duplicate on YPS (yeast extract 0.25 %, peptone 0.25 %, 0.6 M-sucrose, 2 % agar, w/v) containing various concentrations of staurosporine, H-7, cycloheximide or griseofulvin. After culturing at 24 °C for two weeks, the numbers of colonies were counted. More than two independent experiments were done with each compound.

Effect of staurosporine and Polyoxin D on hyphal morphology. Mycelium was grown in 20 ml GMY in a 100 ml flask for 7–10 d, then filtered and washed with distilled water. The mycelium was homogenized and a small amount transferred to 1 ml GMY containing $0.25 \mu\text{g ml}^{-1}$ ($0.54 \mu\text{M}$) of staurosporine or $5 \mu\text{g ml}^{-1}$ ($9.5 \mu\text{M}$) polyoxin D and grown at 24 °C. After 2 d, hyphal morphology was examined by microscopy.

Fig. 1. Inhibition of mycelial growth of *P. ostreatus* by staurosporine. Mycelium was grown in duplicate on MA containing various concentrations of staurosporine. The inhibition ratio of mean linear growth against the control was calculated. This is one of two independent experiments.

Fig. 2. Effect of staurosporine on the regeneration of protoplasts of *P. ostreatus*. Protoplasts ($5 \times 10^6 \text{ ml}^{-1}$) were plated on YPS containing various concentrations of staurosporine in duplicate. Regeneration frequency was determined by the mean number of colonies regenerated against the number of colonies on YPS without staurosporine. The absolute regeneration frequency in controls was 0.1 %. These results are from one of two essentially identical experiments.

Fig. 3. Effect of polyoxin D and staurosporine on the morphology of mycelium of *P. ostreatus* grown in GMY, and photographed 48 h after addition of the antibiotics under the conditions described in Methods. (a) Control; (b) $9.5 \mu\text{M}$ polyoxin D; (c) $0.54 \mu\text{M}$ staurosporine.

Results

Effect of staurosporine on growth of mycelia

The growth rate of K01-M2 was 0.35 cm d^{-1} when staurosporine was not included in MA. The inhibition by staurosporine of the linear growth of the colony correlated with its concentration (Fig. 1). Mycelial growth was completely inhibited at $9.6 \mu\text{M}$ -staurosporine. Staurosporine did not cause the colony to be flat or sparse; only the linear expansion of the colony was disturbed.

Effect of staurosporine on regeneration of protoplasts

Regeneration frequency was determined by comparing the number of colonies on YPS that contained staurosporine to those regenerated on YPS only. Then the inhibitory effect of each concentration of staurosporine was calculated against the regeneration frequency on YPS. The absolute regeneration frequency without staurosporine was 0.1 %. Regeneration of protoplasts was completely inhibited above $6.5 \mu\text{M}$ -staurosporine (Fig. 2).

Another protein kinase inhibitor, H-7 (Hidaka *et al.*, 1984), was much less effective on protoplasts. At $100 \mu\text{M}$, regeneration of protoplasts was not inhibited, and at $250 \mu\text{M}$, inhibition was only 63 %.

Similar experiments were done with cycloheximide and griseofulvin, which had also been found to show strong growth inhibition of protoplasts of *P. ostreatus*. The MICs for griseofulvin and cycloheximide were 200 μM and 25 μM , respectively; much greater than that of staurosporine.

Effect of staurosporine on hyphal morphology

At 0.54–0.96 μM -staurosporine, periodic constrictions appeared in the mycelium, frequency-dependent on the concentration. Staurosporine caused swellings of hyphal tips and less often of subapical regions (Fig. 3c) while formation of stubby, bulbous hyphae was also observed.

In contrast, swelling induced by polyoxin D appeared strictly at the tips of hyphae (Fig. 3b). The width of hyphae was not altered by polyoxin D.

Discussion

Staurosporine is known as a strong inhibitor of protein kinases. Its reported IC_{50} values for mammalian protein kinases are 2.7 nM for PKC, 8.2 nM for cAMP-dependent protein kinase and 6.4 nM for protein tyrosine kinase. Staurosporine also shows a strong cytotoxic effect on the growth of mammalian cells (IC_{50} values: < 3 nM for HeLa S3 cells, 90 nM for MCF-7 cells) (Tamaoki, 1991). In the present study, it is clear that this antibiotic inhibited the growth of both the protoplast (MIC 6.5 μM) and the mycelium (MIC 9.6 μM) of *P. ostreatus* at low concentration. These values were 10^2 to 10^3 times greater than the IC_{50} for mammalian cells, but smaller than the MICs for *Candida albicans* (6.25 $\mu\text{g ml}^{-1}$, 13.4 μM) and *Aspergillus niger* (25 $\mu\text{g ml}^{-1}$, 53.6 μM) (Omura *et al.*, 1977). Furthermore, its inhibitory effect was stronger than that of two of the antibiotics which we found to be very potent inhibitors of protoplast regeneration in *P. ostreatus*. Our experiments suggest that protein kinases may regulate events essential for the growth of the fungus.

The difference between the MIC for protoplasts and mycelium was only slight, indicating that the cell wall was not a barrier to staurosporine. It is important for antibiotics and pharmacological agents to be effective at as low a concentration as possible to avoid non-specific interactions.

A synthetic protein kinase inhibitor, H-7 (Hidaka *et al.*, 1984), whose K_i value for PKC is 6.0 μM , was ineffective on *P. ostreatus* protoplasts at much higher concentration. Based upon the K_i value, a concentration of H-7 in the mM range might be necessary to inhibit the protein kinase of *P. ostreatus* *in vivo*, and therefore H-7 is not a suitable inhibitor for the protein kinases of this fungus.

Staurosporine had not previously been reported to

cause morphological alterations in fungi. Here we found that a sublethal concentration (0.54 μM) induced morphological changes in *P. ostreatus* hyphae. To examine how cell wall biosynthesis might be involved, comparison was made with morphological changes induced by a chitin synthase inhibitor, polyoxin D. Polyoxin D from *Streptomyces cacaoi* is a strong competitive inhibitor of chitin synthetase in fungi (Isono *et al.*, 1967; Endo *et al.*, 1970) which inhibits, for example, the enzyme from *Mucor rouxii* with a K_i value of 0.6 μM (McMurrough & Bartnicki-Garcia, 1971). At 19 μM to 190 μM , polyoxin D inhibits the germination and growth of *M. rouxii* (Bartnicki-Garcia & Lippman, 1972). In our study, polyoxin D induced ballooning strictly at the hyphal apex of *P. ostreatus* (Fig. 3b). The hyphal apex is the region where cell wall biosynthesis is most active in *Schizophyllum commune* (Gooday, 1971; Gooday & Trinci, 1980; Wessels *et al.*, 1983; Sietsma *et al.*, 1985), and probably in *P. ostreatus*. Although a similar type of swelling of hyphal tips was induced by staurosporine (Fig. 3c), others occurred at the subapical regions. Other than swelling, mycelium became shorter and wider, a phenomenon not observed with polyoxin D. The response to staurosporine resembled that described by Schmid & Harold (1988) accompanying a decrease in the extracellular Ca^{2+} concentration, in which the hyphae of *N. crassa* became wider as well as shorter.

The results described here show for the first time that cell wall biosynthesis is affected by staurosporine, which serves in certain systems as an inhibitor of protein kinase(s). Since modification of the hyphal morphology of *P. ostreatus* by staurosporine was actually more complex than that caused by polyoxin D, however, chitin biosynthesis is not likely to be the only event regulated by the fungal protein kinases(s).

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