Purification and characterization of protein kinase C from *Pleurotus ostreatus*

YUMI MAGAE*

National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki, Japan 305

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Protein kinase C (PKC) from *Pleurotus ostreatus* mycelium (Basidiomycotina) was purified by affinity chromatography using activated agarose (Affigel 15) coupled with the protein kinase inhibitor *N*-(2-aminoethyl)-5-isoquinolinesulphonamide hydrochloride. Using buffer containing 2.0 M-arginine. PKC activity eluted as a single peak corresponding to a protein with apparent molecular mass of approximately 68 000 Da as estimated by SDS-PAGE. The enzyme was not stimulated by Ca^{2+} alone, and activation by phosphatidylserine (PS) was significant only at 10^{-6} M- Ca^{2+} . However, the activity was enhanced by the addition of 1,2-dioctanoyl-*sn*-glycerol in the presence of PS and Ca^{2+} . Additionally, although sensitivity was lower than for mammalian PKC, the enzyme was activated by a tumour-promoting phorbol ester, phorbol-12-myristate 13-acetate (PMA). The saturation concentration of PMA was $50 \mu\text{g ml}^{-1}$, which was 5×10^3 times the value for mammalian PKC.

Introduction

Pleurotus ostreatus, in common with other basidiomycetes, responds to environmental factors such as low temperature (Eger, 1976), light (Zadrazil, 1978), and nutrients (Eger, 1968) to differentiate basidiocarps, and it is likely that this differentiation involves signal transduction pathways.

With *Schizophyllum commune*, increased cAMP content on exposure to UV-A light has been reported (Yli-Mattila, 1987), and similarly increased cAMP content and adenylate cyclase activity have been reported in *Lentinus edodes* at primordium formation (Takagi *et al.*, 1988). These studies suggest that cAMP may act as a second messenger at the point of fruiting body formation. However, there are as yet no detailed studies on the molecular mechanism of basidiocarp differentiation.

Currently, 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol, which are produced by the turnover of membrane inositol phospholipids, are known to act as messengers in mammalian cells (Takai *et al.*, 1979). Since

protein kinase C (PKC) is activated by diacylglycerol, and also by a tumour-promoting phorbol ester phorbol-12-myristate 13-acetate (PMA) (Costagna *et al.*, 1982), this enzyme has attracted much interest. There have also been numerous reports documenting the involvement of PKC in cellular responses, including cell proliferation and differentiation in mammalian cells (reviewed by Nishizuka, 1986; Fargo & Nishizuka, 1990).

In fungi, there are only two reports so far on enzymological work with PKC. Ca^{2+} /phospholipid-dependent protein kinase activity was detected in DEAE-cellulose fractions of *Neurospora crassa* (Favre & Turian, 1987), but the activity was not activated by diacylglycerols or PMA. PKC found in *Saccharomyces cerevisiae* was sensitive to diacylglycerol, but was not activated by PMA (Ogita *et al.*, 1990). Fungal protein kinase activated by PMA has not yet been described.

Among the chemical protein kinase inhibitors, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) is reported to be effective on PKC (Hidaka *et al.*, 1984; Kawamoto *et al.*, 1984). In their subsequent work, its amino derivative, *N*-(2-aminoethyl)-5-isoquinoline sulphonamide (H-9) bound to CNBr activated sepharose was used in affinity chromatography for purification of PKC from rat brain (Inagaki *et al.*, 1985, 1987).

In the present work, H-9 was coupled to activated agarose beads (Affigel 15) and used to isolate PKC from *P. ostreatus*. The properties of the enzyme were then assessed.

Author for correspondence. Tel. 0298 38 8046; fax 0298 38 7996.

Abbreviations: PKC, Ca^{2+} /phospholipid-dependent protein kinase; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; H-9, *N*-(2-aminoethyl)-5-isoquinolinesulphonamide hydrochloride; DOG, 1,2-dioctanoyl-*sn*-glycerol; PS, phosphatidylserine; PMA, phorbol-12-myristate 13-acetate; IP_3 , inositol 1,4,5-trisphosphate.

Methods

Chemicals. H-9 and phosphatidylserine were purchased from Seikagaku Kogyo and Affigel 15 from Bio-Rad. Histone H1 (type III-S), 1,2-dioctanoyl-*sn*-glycerol (DOG) and PMA were from Sigma. [γ - 32 P]ATP was from New England Nuclear. All other chemicals were of analytical grade.

Organism and media. A dikaryotic strain, *Pleurotus ostreatus* K01 was obtained from the Kawamura Edible Mushroom Laboratory. This strain is able to differentiate basidiocarps at temperatures above 18 °C and is compatible with *P. ostreatus* ATCC 60691 (Magae *et al.*, 1990). *P. ostreatus* K01 was grown in 500 ml flasks containing 100 ml of GMY (glucose 1% (w/v), malt extract 1% (w/v), yeast extract 0.4%, adjusted to pH 6.0) at 24 °C, static for 17 d in darkness, then at 18 °C with 100 lux light every 12 h for 5 d.

Preparation of affinity chromatography column. H-9-bound agarose was prepared by incubating 5 mg H-9 (324.2 Da) dissolved in 2.5 ml 0.1 M-NaHCO₃ (pH 8.0) with 2.5 ml of swollen Affigel 15, overnight at 4 °C with constant agitation. Remaining reactive groups were blocked by adding 0.25 ml 1 M-ethanolamine-HCl (pH 8.0) with further agitation for 1 h. The gel (2.5 ml) was then packed into a polypropylene column (0.75 × 5.8 cm) washed with 20 column vols of 0.1 M-NaHCO₃, and equilibrated with the extraction buffer.

Purification of PKC. Mycelium was harvested by filtration, rinsed thoroughly with distilled water, and immediately frozen in liquid nitrogen. Frozen mycelium (100 g weight) was suspended in 100 ml extraction buffer (25 mM-Tris/HCl (pH 7.5), 10 mM-EGTA, 5 mM-EDTA, 0.3% β -mercaptoethanol, 50 μ M PMSF ml⁻¹, 10 mM-benzamidine, 0.02% leupeptine), and homogenized. The homogenate was centrifuged for 20 mins at 30000 *g* and the resultant supernatant further centrifuged at 100000 *g* for 1 h. The supernatant (170 ml, A_{280} = 0.53) was directly applied to the H-9 affinity column (bed volume 2.5 ml), washed with 20 vols buffer (50 mM-Tris/HCl, pH 7.5, 1 mM-EGTA, 2 mM-EDTA, 0.3% β -mercaptoethanol, 50 μ M PMSF ml⁻¹) containing 1.0 M-NaCl. PKC was eluted by a single-step elution with 2.0 M-arginine in 25 mM-Tris/HCl (pH 7.5) containing 50 μ M PMSF ml⁻¹ at a flow rate of 0.3 ml min⁻¹, and 2.5 ml fractions were collected.

Protein kinase assay. The enzyme activity was determined by measuring the incorporation of 32 P into histone from [γ - 32 P]ATP. The standard assay mixture (200 μ l) contained buffer A (50 mM-Tris/HCl, pH 7.5, 5 mM- β -mercaptoethanol), 0.5 mg histone H1 ml⁻¹ (type III-S), 0.1 mM-CaCl₂, 50 μ M PS ml⁻¹, 10 μ M DOG ml⁻¹, 10 mM-Mg (CH₃COO)₂, 150 μ M-ATP containing 0.5 μ Ci (18.5 kBq) [32 P]ATP. PS and DOG were dissolved first in a small amount of chloroform and dried under nitrogen. The residue was then resuspended in buffer A by vortexing and sonicated for 3 min. Additionally, basal protein kinase activity was determined with assay mixture containing 5.0 mM-EGTA without CaCl₂, PS and DOG. Ten microlitres of each fraction of the H-9 column was assayed.

After incubation for 10–30 min at 30 °C, the reaction was stopped by the addition of 1.0 ml 25% (v/v) TCA. For the detection of PKC fraction, the precipitates were filtered on a 96-well filtration plate (PVDF, pore size 0.45 μ m, Millipore) and washed three times with 400 μ l 25% (v/v) TCA. For the characterization of PKC, the CaCl₂ concentration was varied as indicated in each experiment. PMA was dissolved in methanol to give 5 mg ml⁻¹ and was diluted with buffer A immediately prior to use. The TCA precipitates were filtered on to 25 mm nitrocellulose membrane filters (Advantec, pore size 0.45 μ m) and washed (× 3) with 5.0 ml of 25% (v/v) TCA. Filters were dried and radioactivity was determined by liquid scintillation spectrometry by Cerencov counting or as otherwise stated.

SDS-PAGE. Proteins were electrophoresed by the method of Laemmli (1970) in 0.1 × 10 × 10 cm 12.5% (w/v) acrylamide precast gel (Seikagaku Kogyo) and silver-stained. Apparent molecular mass was calculated from a plot for the following marker proteins (Bio-Rad low molecular mass standards): phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; soybean trypsin inhibitor, 21 kDa.

Results

Purification of PKC

H-7 is a competitive inhibitor of protein kinases which interacts with the ATP binding domain, and H-9 is the amino derivative of H-7. The affinity chromatography matrix used in this study was activated agarose, which has an *N*-hydroxysuccinimide ester at the end of a 15-atom spacer arm. The *N*-hydroxysuccinimide ester immediately couples to ligands that contain a primary amino group in the pH range 6.5–8.5. The amount of H-9 in the reaction mixture before and after immobilization was determined by the A_{227} of H-9 at pH 2. The coupling efficiency was 1.10 ± 0.2 mg H-9 (3.4 ± 0.6 μ mol H-9) per ml gel. The H-9-bound Affigel 15 was highly stable and gave reproducible results in repeated use for at least 10 runs.

The total protein bound to an H-9 gel (2.5 ml volume) was calculated from the A_{280} , after washing with the buffer containing 1.0 M-NaCl, and was approximately 0.02% of that present in the 100000 *g* centrifuge supernatant. The protein concentration (A_{280}) of the PKC fraction was undetectably low. Furthermore, since arginine interacts with Coomassie brilliant blue and develops blue colour, the protein concentration of the

Fig. 1. Elution profile of *P. ostreatus* PKC from the H-9 column. Fractions were assayed as described in Methods, in the presence of ○, 0.1 mM-CaCl₂, 50 μ M PS ml⁻¹ and 10 μ M DOG ml⁻¹; ●, 5.0 mM-EGTA. Fractions corresponding to peaks A, B and C were used for electrophoresis.

Table 1. The purification of PKC of *P. ostreatus* by H-9 affinity chromatography

Sample	Protein kinase activity (c.p.m. ml ⁻¹)		A/B	Volume (ml)	Total activity (c.p.m.)	Yield (%)
	A*	B†				
100 000 g supernatant	170	600	0.28	170	28 900	100
H-9 Fraction 19	1290	270	4.8	2.5	3225	11

* Protein kinase activity determined in the presence of 0.1 mM-Ca²⁺, 50 µg PS ml⁻¹ and 10 µg DOG ml⁻¹.

† Protein kinase activity determined in the presence of 5.0 mM-EGTA.

active fraction could not be measured by the method of Bradford (1976). Therefore, protein concentrations of H-9 fractions could not be accurately determined.

When buffer containing 2.0 M-arginine was applied to the H-9 column, high protein kinase activity in the presence of EGTA appeared in the first five fractions (Fig. 1, peak A). Protein kinase activity dependent on Ca²⁺, PS and DOG eluted at between 19 and 20 column vols (Fig. 1, peak C). This elution profile of the H-9 agarose affinity chromatography was highly reproducible, essentially giving the same result in each experiment.

Since the accurate protein concentration of fractions 19 and 20 was not known, purification factors for calculating specific activity could not be determined. However, the ratio of the radioactivity values (c.p.m.) for assay A and assay B (A/B in Table 1) provided an estimate of the purity of PKC. As shown in Table 1, the value for the starting material was 0.28, whereas that of fraction 19 was 4.8. An approximately 17-fold purification was achieved by the H-9 column.

Fraction 19 was used for all subsequent assays.

Electrophoresis of H-9 fractions

Fractions corresponding to peaks A, B and C (Fig. 1) were concentrated 100-fold by ultrafiltration and subjected to SDS-PAGE (Fig. 2). Although two proteins with lower molecular masses (indicated in Fig. 2 by dots) were present in every fraction, a band of molecular mass 68 kDa occurred in the active fraction (Fig. 2, C). This protein appeared to correspond to protein kinase C activity in other experiments also.

Activation by diacylglycerol and phorbol ester

One of the properties of mammalian PKC is that it is activated by diacylglycerol. Firstly, with the PKC from *P. ostreatus* K01, protein kinase activity was assayed in

the presence of 0.5 mg PS ml⁻¹, 10⁻⁴ M-CaCl₂ for 20 min at 30 °C. The concentration of DOG added was 0.1 mg ml⁻¹. ³²P incorporated into histone was measured by liquid scintillation counting. The results of duplicate assays were 12 364 ± 333 c.p.m. in the presence of DOG, and 1092 ± 279 c.p.m. without DOG. This established that *P. ostreatus* PKC was activated by DOG in the range 0.001–1 mg PS ml⁻¹ (data not shown).

Tumour-promoting esters such as PMA and PDBu can substitute for diacylglycerol and activate mammalian PKC in the presence of Ca²⁺ and PS (Costagna *et al.*, 1982). When the concentration of PMA was varied, activation was observed (Fig. 3). Saturation concentration of PMA for PKC from *P. ostreatus* was 50 µg ml⁻¹.

Ca²⁺ requirements

Activity was measured using various concentrations of Ca²⁺ (Fig. 4). PKC from *P. ostreatus* was insensitive to Ca²⁺ alone, but with both PS and PMA present, activity was enhanced at lower concentrations (up to 10⁻⁶ M) of Ca²⁺.

Discussion

To directly prove that PKC is present in *Pleurotus*, affinity chromatography using H-9 was chosen as the purification method rather than the conventional DEAE-cellulose procedure. This single-step chromatographic procedure successfully isolated PKC. Protein kinase bound to H-9 is usually eluted with either glycerol or arginine (Inagaki *et al.*, 1985). The concentration of arginine necessary for the elution of PKC from *P. ostreatus* was 2.0 M, which was much higher than that needed for the elution of rat brain PKC (0.7 M).

However, H-9 affinity chromatography has a drawback. Protein kinase activity in 2.0 M-arginine fractions was not retained at -80 °C, and when arginine was removed, protein kinase activity immediately ceased. It

Fig. 2. SDS-PAGE patterns of H-9 fractions. Fractions A, B and C (volume 2.5 ml), from peaks shown in Fig. 1, were concentrated to 20 μ l, subjected to electrophoresis in a 12.5% (w/v) SDS-polyacrylamide gel and silver-stained. The positions and apparent molecular masses of the protein size standards are indicated. See text for significance of dots.

Fig. 3. Activation of PKC by PMA. Protein kinase activity was determined with the standard assay mixture as described in Methods with various concentrations of PMA in place of DOG. The data shown are means and standard deviations from duplicate determinations of four independent experiments (Δ , $\square$, $\blacksquare$, $\bullet$). Control activity (100%) was 1183 ± 88 c.p.m. attained in the presence of 10 μ g DOG ml^{-1} .

was impossible to achieve long-term preservation of the active PKC. Protein kinase activity of H-9 fractions in the presence of EGTA gradually increased while kept at 4 °C. Presumably, degradation to the catalytic and regulatory domains occurred at high arginine concentrations. Therefore, all the results in this study are from the experiments carried out within two days after

Fig. 4. Activation of PKC at various concentrations of CaCl_2 . PKC was assayed as described in Methods. $\bullet$, In the absence of PS and PMA; Δ , in the presence of 50 μ g PS ml^{-1} , $\circ$, in the presence of 50 μ g PS ml^{-1} and 50 μ g PMA ml^{-1} . Control activity (100%) was 1120 ± 240 c.p.m. attained in the presence of 10^{-6} M- CaCl_2 , 50 μ g PS ml^{-1} and 10 μ g DOG ml^{-1} . Data are means and standard deviations from duplicate determinations and are representative of data obtained from PKC purified on three independent occasions.

the elution from the H-9 column, while the protein kinase activity was still affected by EGTA.

Two proteins having a high affinity for H-9 were found, which were relatively abundant in the cytosol fraction of *P. ostreatus*. Their apparent molecular masses were 16 and 18 kDa (Fig. 2a, b, c, indicated by dots). Similar proteins from *Coprinus cinereus* have also been detected in the 2.0 M-arginine fractions from an H-9 column (data not shown).

In the present study, the amount of PKC recovered in the cytosol fraction derived from 100 g frozen *P. ostreatus* mycelium was in the nanogram range and could be revealed by silver staining or very slightly with Coomassie brilliant blue. The amount of enzyme in the membrane fraction was not examined.

PKC from *P. ostreatus* was not as dependent on Ca^{2+} as that of *N. crassa*. The *N. crassa* PKC showed a steep increase in activity with the addition of micromolar amounts of Ca^{2+} (Favre & Turian, 1987), whereas *P. ostreatus* PKC showed a slight increase in activity dependent on Ca^{2+} concentration (Fig. 4). The most distinctive property of *P. ostreatus* PKC is that, although lower in sensitivity than mammalian PKC, it displayed activation on addition of PMA. PKCs from *N. crassa* and *Saccharomyces cerevisiae* are both insensitive to PMA (Favre & Turian, 1987; Ogita *et al.*, 1990). A comparison of DNA sequences of PKC among these fungi might elucidate the differences between the diacylglycerol and PMA sensitivities.

IP_3 has recently been identified in *N. crassa* (Hanson,

1991). This suggests the possibility of phospholipase-mediated signal transduction in fungi. Nevertheless, the external stimuli which activate PKC in *P. ostreatus* are at present unknown. Mechanisms in which PKC functions as a mediator of external stimuli, possibly with phospholipid turnover, remain to be elucidated.

Staurosporine, which is a potent inhibitor of PKC, was recently found to alter hyphal morphology of *P. ostreatus* at very low concentrations (Magae & Magae, 1993). A search for endogenous substrates of PKC and an examination of the possible involvement of PKC in basidiocarp morphogenesis is in progress.

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