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# PURIFICATION AND CHARACTERIZATION OF A PROTEIN KINASE FROM PINE POLLEN

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**Key Word Index**—*Pinus canariensis*; Pinaceae; pine; cyclic AMP; pollen; protein kinase; protein phosphorylation.

**Abstract**—A kinase phosphorylating casein and phosvitin has been purified from pine pollen by a three-step procedure involving DEAE-cellulose chromatography, affinity chromatography on casein-Sepharose and Sephadex G-100. A purification of about 2000 fold was obtained by this procedure. The kinase is affected neither by cyclic nucleotides nor by $Ca^{2+}$-calmodulin, whereas it is strongly inhibited by heparin. Using this purification procedure, we have isolated protein kinase exhibiting phosphorylating activity towards casein in the pollen of many other Pinaceae species.

## INTRODUCTION

The process of protein phosphorylation–dephosphorylation, catalysed by protein kinase (EC 2.7.1.37) and phosphoprotein phosphatase (EC 3.1.3.16) systems, represents an important mechanism in controlling many biochemical and physiological processes of the animal cells [1]. Cyclic AMP and $Ca^{2+}$ play their second messenger role through the modulation of cyclic AMP-dependent protein kinases and calmodulin-dependent protein kinases respectively [1, 2].

In higher plants, phosphorylation by kinase has been reported for many proteins, including nuclear and ribosome-associated proteins [3–12], thylakoid proteins [13, 14], membrane components [15] and cytokinin-binding protein [16]. Two enzymes, pyruvate dehydrogenase and quinate: $NAD^+$ oxidoreductase, and wheat-germ initiation factor eIF-2 have been reported to be regulated by phosphorylation [17–19]. Although there is convincing evidence that cyclic AMP exists in higher plants [20] and cyclic AMP binding protein has been detected in several plant species [20, 21] the protein kinases which have been so far isolated from higher plants appear to be independent of cyclic AMP ([22–24] and for review [20]). However, a recent paper [25] reports the isolation of a cyclic AMP-dependent histone kinase from *Lemna*. The presence of the $Ca^{2+}$-binding protein, calmodulin, as well as calmodulin-dependent protein kinases in higher plant tissues have also been described recently [26–30].

In a previous paper [31] we reported the presence of protein kinase activity in extracts of pine pollen, giving the first experimental evidence for the presence of protein kinase in a gametophytic tissue. DEAE-cellulose chromatography resolved this activity in two peaks. One, eluting with 0.1 M potassium chloride, was most active towards casein and did not exhibit endogenous activity, whereas the other, eluting with 0.2 M potassium chloride, phosphorylated an endogenous substrate, in addition to casein and histone. The present paper deals with the purification and characterization of the first peak. Purification of the other peak and its natural substrate is under investigation.

## RESULTS AND DISCUSSION

### *Purification of the protein kinase activity*

The results of a typical purification of the pine pollen protein kinase are summarized in Table 1. A purification of 2035 fold was obtained with this procedure. Affinity chromatography on casein-Sepharose proved to be the key step in the purification protocol, giving the highest increase in enzyme specific activity.

The purity of the protein kinase preparation was

Table 1. Purification of protein kinase from pine pollen

| Step of purification | Total protein (mg) | Specific activity (p mol $^{32}P$ incorp./ 2 min/mg protein) | Purification (fold) |
|---|---|---|---|
| 105 000 *g* supernatant after desalting on Sephadex G-25 | 200 | 20 | 1 |
| DEAE-cellulose column chromatography | 2.45 | 505 | 25 |
| Casein-Sepharose affinity chromatography | 0.054 | 16 900 | 844 |
| Sephadex G-100 column chromatography | 0.018 | 40 700 | 2035 |

Protein kinase activity was assayed under standard condition with dephosphorylated casein as substrate. Before assaying, the enzyme fractions from each purification step were dialysed overnight against buffer A to eliminate KCl.

assessed by analytical disc gel electrophoresis. A single protein-staining band was observed and it was coincident with the protein kinase activity. The purified enzyme was devoid of cyclic AMP phosphodiesterase and cyclic AMP binding activity.

*Enzyme properties*

The protein kinase exhibited a pH optimum between 7.2 and 7.8. 50% of maximum activity was observed at pH values of 5.8 and 8.8. The enzyme activity had an absolute requirement for $Mg^{2+}$ with maximal activity being obtained at 8 mM; higher concentrations of $Mg^{2+}$ were inhibitory. Potassium chloride and sodium chloride were ineffective up to 10 mM and inhibitory at higher concentrations (50% inhibition at 40 mM).

The apparent MW of the protein kinase was 48 000 ± 2700 (± SD from three estimations) as determined from gel filtration on Sephadex G-100. SDS-electrophoresis of the purified kinase revealed a single band (MW 47 000 ± 1000). These data indicate that the protein kinase has an MW of *ca* 47 000 and is composed of one subunit.

*Substrate specificity*

In reaction mixtures containing 5 μg purified enzyme and 1 mg/ml of exogenous substrate, the enzyme phosphorylated casein and phosphvitin (203 and 164 pmol $^{32}P$/min, respectively). However, the enzyme did not phosphorylate histone, protamine or bovine serum albumin. In addition, we found that the protein kinase had no activity towards the purified endogenous substrate phosphorylated by the kinase eluting from DEAE-cellulose at 0.2 M potassium chloride [31] as well as towards membrane fractions isolated from pine pollen (data not shown).

The $K_m$ values of the enzyme for casein in the standard assay conditions at pH 7.6 was 560 μg/ml. The $K_m$ for ATP in the standard assay conditions with 1 mg/ml of casein was 8 μM as determined from Lineweaver–Burk analysis.

*Effect of various compounds on the protein kinase activity*

Whatever the substrate used, no stimulation of the enzyme by the following compounds (tested at $10^{-6}$ M and $10^{-5}$ M) was observed: cyclic AMP, cyclic GMP, cyclic IMP, adenosine, isopentenyladenosine and 5′-AMP. The enzyme was also not inhibited by the protein inhibitor of animal cyclic AMP-dependent protein kinase [32] nor by cyclic AMP binding protein isolated from Jerusalem artichoke [33] when these were included in the standard assay at 1 mg/ml. Thus, this gametophytic protein kinase appears to be insensitive to cyclic AMP, as observed for the many protein kinases isolated from sporophytic tissues ([22–24] and for review [20]). In addition, we found that $Ca^{2+}$ (up to 200 μM) did not affect the enzyme activity in the presence or absence of calmodulin (up to 100 μM).

Since heparin has been demonstrated to strongly inhibit cyclic AMP-independent casein kinases from animal tissues [34–36] and wheat germ [23], we tested the effect of this substance on the pine pollen protein kinase. Likewise, we found that this enzyme activity was also strongly inhibited by heparin (60% inhibition at 1 μg/ml and 90% at 15 μg/ml).

*Presence of protein kinase in the pollen of other Pinaceae species*

With the purification procedure described in this paper, we isolated protein kinase, exhibiting phosphorylating activity towards casein, from the following Pinaceae species: *Pinus pinea* L., *Pinus pinaster* Sol., *Picea excelsa* Lk., *Abies alba* Miller., *Thuja orientalis* L., *Cupressus sempervirens* L. and *Taxus baccata* L. This suggests that the enzyme is ubiquitous in pollen of Pinaceae species.

## EXPERIMENTAL

*Materials.* All organic unlabelled substances were obtained from Sigma except for nucleotides which were from Boehringer. ATP($\gamma$-$^{32}P$) (sp. act. 17 Ci/mmol) was obtained from Amersham. Dephosphorylated casein (from Sigma) was coupled to cyanogenbromide-activated Sepharose 4 B according to the instructions of the manufacturer. Calmodulin was purified from sheep brain by a procedure involving heat-treatment (85° for 5 min) and affinity chromatography on a CAPP-Sepharose 4 B matrix [37].

*Plant materials.* Pollen was collected from pines (*Pinus canariensis* P. Sm) growing at the botanical garden of Portici. It was stored dry under vacuum at −20° for many years without loss of vitality.

*Protein kinase activity.* This was determined as previously reported [10, 31] using ATP ($\gamma$-$^{32}P$) as a phosphate donor. Each reaction mixture contained in a final vol. of 0.2 ml: 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 10 μM ($\gamma$-$^{32}P$) ATP (170 000 cpm/nmol), 200 μg protein substrate and 20 μl of enzyme soln. The incubation was conducted at 37° for 2 min. Unless stated otherwise, dephosphorylated casein was used as exogenous substrate.

*Effect of cyclic nucleotides and $Ca^{2+}$-calmodulin on the protein kinase activity.* Protein kinase activity was assayed under standard conditions except that cyclic nucleotides (cyclic AMP, cyclic GMP and cyclic IMP) and other substances were added to the reaction mixtures.

*Other methods.* Cyclic AMP phosphodiesterase and cyclic AMP binding activity were determined as previously reported [33]. Analytical disc gel electrophoresis was carried out as previously reported [33] using 7% acrylamide, SDS-polyacrylamide gel electrophoresis was conducted as described by [23]. The MW markers used were phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000) and cytochrome *c* (17 200). Protein concn was determined by the method of ref. [38] using BSA as standard.

*Purification of protein kinase activity.* All procedures were carried out at 4°. The initial steps of purification were essentially identical to those previously reported [31] except that 10 g of pine pollen were used routinely for each preparation. The 105 000 *g* supernatant containing 200 mg of protein was loaded onto a column of DEAE-cellulose Whatman DE-52 (2.6 × 15.4 cm) which had been previously equilibrated with 25 mM Tris–HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ (buffer A). Following adsorption of the protein, the column was thoroughly washed with 250 ml of buffer A and eluted with buffer A containing 0.1 M KCl (buffer B). Fractions of 5 ml were collected. The eluate containing protein kinase (40 ml) was incubated with 10 ml bed vol. of casein-Sepharose (about 3.5 mg casein bound/g wet weight of gel) in the buffer B. The suspension was kept under agitation for 2 hr and then transferred within a column to pack the gel. After extensive washing of the affinity gel with buffer B, protein kinase was eluted with buffer A containing 0.5 M KCl (buffer C). The eluate, exhibiting protein kinase activity, was concd by Amicon ultrafiltration (PM 10 membranes) to 5 ml and

chromatographed subsequently on Sephadex G-100. A column of Sephadex G-100 (2.1 cm × 54 cm), previously equilibrated with buffer C, was loaded with the 2.5 ml aliquots from Sephadex fraction and eluted with the same buffer at a flow rate of 5 ml/hr. Fractions of 2.5 ml were collected. Protein kinase activity emerged as a single peak eluting between 85 and 100 ml. The active fractions were pooled, dialysed against buffer B, concd by Amicon ultrafiltration (PM 10 membrane filter) to 0.1 ml and stored at 4°. No loss of activity was observed after 1 week.

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