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ISOLATION OF cAMP-INDEPENDENT HISTONE KINASE FROM PINE COTYLEDONS

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Abstract—A cyclic AMP-independent histone kinase was extracted and partially purified from growing cotyledons of *Pinus pinea*. The enzyme was purified 270-fold by $(NH_4)_2SO_4$ precipitation and chromatography on Sephadex G-25 and DEAE-Sephadex A-50. The kinase showed maximum activity at pH 7.8 in Tris–HCl buffer and was shown to be Mg-dependent. Enzyme activity was greatly suppressed by the addition of *p*-chloromercuribenzoate and monoiodoacetic acid; however, 2-mercaptoethanol reversed *p*-chloromercuribenzoate inhibition. The enzyme phosphorylated calf thymus histone, *Pinus pinea* histone and protamine but not casein. The addition of exogenous kinetin or cAMP had no stimulatory effect on reaction rate.

INTRODUCTION

There is relatively little information available concerning the isolation and purification of plant histone kinases and less still concerning those obtained from gymnosperms. This is in direct contrast to the evidence on the existence of both cAMP-dependent and -independent protein kinases (EC 2.7.1.37) in animal tissues which enzymatically phosphorylate casein, protamine and/or histone [1–7]. Kuo and Greengard [8], initially reported the absence of cyclic AMP-dependent kinases in plant tissues. It was stated, however, that the evidence was not entirely conclusive since the assay methods employed to study animal protein kinases were not suitable for their detection in plants. Since then, there have been several reports on the mechanism of the regulation of histone modification and histone phosphorylation in higher plants. Keates [9] successfully isolated a cyclic nucleotide-independent protein kinase from pea shoots which phosphorylated casein or phosvitin but not histones. In addition, recent reports show the presence of protein kinases in chinese cabbage and tobacco [10], cAMP binding protein in Jerusalem artichoke [11], *Phaseolus* [12] and barley [13], a lysine-rich histone kinase obtained from soybean [14] and a protein kinase from cultured carrot root cells [15]. Ralph *et al.* [10] postulated from their studies on chinese cabbage leaf discs that cytokinins play the same role in plant cells as does cyclic AMP in animals. Also it was thought that cytokinins are involved in the regulation of ribosomal protein phosphorylation and thus ribosomal activity.

RESULTS

Purification of the protein kinase

Gel filtration of the crude enzyme preparation on Sephadex G-25 revealed the presence of two major protein peaks, the first being a broad fraction of high-MW proteins with slight kinase activity and the second, lower MW fraction, with significant kinase activity. Because the activity contained within the first fraction was minimal, it was not further examined. The second fraction was further purified on DEAE Sephadex A-50 ion exchange resin and was separated into two major fractions: one eluted between 0.18 and 0.22 M NaCl which contained the highest enzymatic activity and a second, larger fraction, eluted between 0.32 and 0.36 M NaCl which contained about 38% of the combined activity of the first peak which was contained in twice the volume of eluant and with higher concentration of protein. Further assays which involved the kinetics, substrate specificity, Mg^{2+} ion, cyclic AMP, kinetin and pH dependencies of the enzyme were made only for the protein fraction having the highest enzymatic activity. Table 1 shows the degree of purification obtained at each purification step.

Enzyme properties

The pH optimum of the kinase against calf thymus histone in Tris–HCl buffer was 7.8 with rapid decline for pH values above or below this value (half maximum pH, 6.8 and 8.5). In Pi buffer the pH optimum was 7.9 and half maximum pH 7.0 and 8.7. *P. pinea* histone kinase activity was stimulated by Mg^{2+}, the optimum concentration of the ion being 6 mM (half maximum concentration 2 mM); concentrations higher than 7.5 mM were inhibitory (half maximum concentration 11 mM). At zero concentration of Mg^{2+} there is about 27% of the maximal activity. Ethylenediamine tetraacetate (EDTA) inhibited enzyme activity (K_i = 0.5 mM). Concentration-dependent decline in enzyme activity occurred down to 0.2 mM EDTA and maximum inhibition (73%) occurred at the highest concentration (20 mM) comparing favorably for enzyme activity in the absence of Mg^{2+}. There was no significant effect on the reaction rate with either cAMP or kinetin using concentrations over the ranges of 10 μM.

In the absence of 2-mercaptoethanol, *p*-chloromercuribenzoate caused a high degree of kinase inhibition (K_i = 0.1 mM). The addition of 1.0 mM *p*-chloromercuribenzoate resulted in complete inhibition but 0.05 mM 2-mercaptoethanol reversed the inhibition; higher concentrations, however, became less effective. Iodo-

Table 1. Purification data from the isolation of *Pinus pinea* histone kinase

	Amount of protein (mg)	Specific activity (pkat/mg protein)	Purification factor (× crude)	Yield (%)
Crude extract	6515	0.011	1.0	100
105 000 g pellet	4796	0.067	5.7	74
35–65% NH_4SO_4	625	1.30	111	9.6
Dialysis	432.4	1.40	120	6.6
Sephadex G-25 (Fraction 2)	144	2.30	197	2.2
DEAE Sephadex S-50 (Fraction 1)	40.8	3.13	268	0.63

acetate is known to affect sulfhydryl groups in much the same way as does *p*-chloromercuribenzoate [16] and was used to demonstrate the presence of essential sulfhydryl groups in the enzyme. Kinase inhibition with iodoacetate required much higher concentrations than *p*-chloromercuribenzoate (K_i = 1 mM).

The temperature optimum and thermal lability of partially purified histone kinase from *P. pinea* was studied. The activation energy under optimal conditions of temperature and pH was 25 kcal/mol and the activation energy of denaturation 35 kcal/mol. Activity was completely lost at 70° but the enzyme has a relatively long half-life (*ca* 3 months) if stored in Tris–HCl buffer with 2-mercaptoethanol at −20°. The half-life is reduced to *ca* 2 days if maintained under similar conditions but at 0–5°.

Enzyme kinetics

The rates of reaction as a function of *P. pinea* histone and calf thymus histone concentration were studied. The value of V_{max} for *Pinus pinea* histone was 4.0×10^{-3} pkat with a resultant K_m value of 40 μg protein/ml. The V_{max} for calf thymus histone was somewhat lower (3.7×10^{-3} p-katals) and the K_m value was 60 μg protein/ml. In addition protamine was found to be a suitable substrate for the kinase but casein was a poor substrate at various concentrations.

DISCUSSION

This is the first report concerning the partial purification and characteristics of a protein kinase from pine (*Pinus pinea*) tissue. Although two fractions of kinase activity appeared following Sephadex G-25 chromatography, only the major peak was further purified on a DEAE-Sephadex A-50 column.

The pH optimum for the purified kinase fraction using calf thymus histone fraction V as substrate was quite similar to that reported for other plant histone kinase [9, 14, 15]. The Mg dependency of the enzyme is clearly demonstrated by both stimulation in the presence of Mg^{2+} and inhibition by EDTA. It is interesting to note that total activity cannot be eliminated by the absence of Mg^{2+} or the presence of EDTA. The optimal concentration of Mg^{2+} coincides with values reported for soybean kinase [14].

The kinase isolated in this study was not significantly affected by either cAMP or kinetin. Although cAMP stimulation for kinase is commonly encountered in enzyme prepared from animal sources, there are very few reports of cAMP-dependent protein kinases in plants [11, 17]. Ralph *et al.* [10] postulated that cytokinins play the same role in plant tissues as does cAMP; however, the *Pinus* kinase fraction is not stimulated by kinetin. It is conceivable that other kinase fraction(s) from the pine tissue may respond to either cAMP or cytokinin.

The presence of essential sulfhydryl groups in the kinase has been demonstrated by the inhibitory action of both *p*-chloromercuribenzene and iodoacetate. These effects were reversed by the sulfhydryl-group protective agent, mercaptoethanol.

EXPERIMENTAL

Purification procedures. A modification of the methods used in ref. [15], for the isolation of histone kinase from carrot root cell culture was used for *P. pinea* cotyledons. All operations were carried out at 0–5°. 25.0 g (fr. wt) of cotyledons at 14 days germination were combined with 10 vol. of 0.05 M Tris–HCl buffer with 0.05 M NaCl and 6 mM 2-mercaptoethanol adjusted to pH 7.8. The tissue was homogenized for 90 sec and the brei was filtered through four layers of 'J-cloth' and then through a series of nylon mesh filters (375, 100, 25 and 10 μm). The filtrate was centrifuged (500 *g*, 10 min, 5°) and the supernatant combined with 15 g Polyclar AT (Serva Biochemicals, Heidelberg, W. Germany) for 10 min and the residue removed by centrifugation (500 *g*, 10 min, 5°). The supernatant was then centrifuged at 105 000 *g* for 60 min at 5°. After completion of this centrifugation step, the crude extract was then combined with Dowex-IX2 (Cl form) which was previously equilibrated with the extraction buffer and was stirred gently for 20 min. The resin was removed by filtration through Whatman No. 4 paper and the protein was then precipitated with $(NH_4)_2SO_4$ and the 35–65% fraction was collected by centrifugation (500 *g*, 10 min, 5°). The precipitate was resuspended in 4.0 ml extraction buffer and dialysed overnight in the same buffer. The resulting precipitate was removed by centrifugation (500 *g*, 10 min, 5°) and discarded.

Approximately 150 mg protein in a 5.0-ml volume was applied to a Sephadex G-25 column (2.5 × 35 cm column) previously equilibrated with extraction buffer adjusted to pH 8.5. The flow-rate was adjusted to 2.3 ml/min and 5.0-ml fractions were collected. Absorbance was monitored at 280 nm. Protein was eluted with 300 ml of buffer. The fraction which showed the greatest kinase activity, (i.e. 230–250 ml) was collected and pooled for further purification. This fraction was then applied to a DEAE-Sephadex A-50 column (1.0 × 15.0 cm) which had been previously equilibrated with 75 ml of the same buffer used for Sephadex G-25. After this step, a linear concentration gradient of NaCl (0.05–0.60 M) was applied using the same buffer. The highest enzyme activity was collected between 0.18 and 0.22 M NaCl.

Assay procedure for histone kinase. The assay procedure was based on the method of Yamamura *et al.* [2]. Enzyme activity was assayed by collecting acid-precipitated protein which had been labelled with adenosine 5′-triphosphate, tetra (triethylammonium) salt, [γ^{32}P] on glass fiber filters. The standard reaction mixture (0.25 ml) contained 50 μg/ml calf thymus fraction V histone, 5 μg/ml kinase preparation, 2.5 M-ATP [γ^{32}P] (0.2 Ci), 3.0 M 2-mercaptoethanol, 5 mM Mg^{2+}-acetate and 5 μM Tris–HCl buffer adjusted to pH 7.5. After incubation at 30° for 5 min, the reaction was halted by the addition of ice-cold 5% TCA with 0.25% sodium tungstate (pH 2.0). The precipitated protein was collected on glass filters under suction and washed extensively with 5% TCA, 0.25% sodium tungstate, Me_2CO, 95% EtOH and finally Et_2O. Radioactivity was assayed by liquid scintillation counting in 0.4% PPO, 0.0% POPOP in toluene.

Assays were carried out to determine pH optimum using 5 μM Tris–HCl (pH 6.5–8.5) and 10 μM K-Pi (pH 7.0–9.0). Also, assays were carried out in the presence of cAMP, 6-furfurylaminopurine, *p*-chloromercuribenzene sulphonic acid and iodoacetic acid.

Pinus pinea histone was isolated from soluble nucleohistone prepared from cotyletons of germinating seeds as described in ref. [18].

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