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PROTEIN KINASE IN CULTURED PLANT CELLS

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Summary

A protein kinase (EC 2.7.1.37) which phosphorylates histones was purified partially from the soluble fractions of cultured plant cells. The optimum pH was 7.5 to 9.0. The activity was not stimulated by exogeneous cyclic AMP. It was thermolabile and completely dependent on the presence of Mg^{2+} or Mn^{2+} for activity. *p*-Chloromercuribenzoate inactivated this enzyme and this inactivation was overcome by mercaptoethanol.

Introduction

Protein phosphorylation by cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase (EC 2.7.1.37) has been clearly demonstrated as a mechanism for the regulation of cellular metabolism which is mediated by cyclic AMP. Protein kinases were found to be present in a variety of animal tissues [1—8] and microorganisms [9] and to be stimulated by the addition of cyclic AMP. However, attempts to find cyclic AMP-dependent protein kinase activity in several plant tissues were unsuccessful [10]. Recently the presence of protein kinase [11,12] and a cyclic AMP-binding protein [13] has been demonstrated in higher plants. In this communication, we wish to report the evidence that a protein kinase which phosphorylates histone is present in carrot cultured cells in suspension culture and to show some properties of the protein kinase.

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Materials and Methods

Materials. [γ-^{32}P] ATP (sodium salt in 50% aqueous ethanol, 1.449 Ci/mmol) was the product of the Radiochemical Center (England). Protamine and casein were purchased from Sigma Chemical Company. The plant material used was strain GD-2 which had been derived from carrot root [14]. Cells were cultured in a synthetic medium of Murashige and Skoog [15]. Logarithmically growing cells were harvested and suspended in 10 vol. of 0.05 M Tris · HCl buffer (pH 7.8). The cells were disrupted in a mortar with quartz sand. The supernatant from centrifugation for 60 min at 105 000 × *g* was used as crude protein kinase preparations.

Cyclic-AMP binding assay. Cyclic-AMP binding was determined by isolation of the receptor-cyclic AMP complex on cellulose-ester membrane filters [16].

Assay procedures of protein kinase. The assay of protein kinase, with histone from calf thymus as the substrate, was based on the method of Yamamura et al. [17]. The reaction mixture (0.25 ml) contained: Tris · HCl buffer (pH 8.6), 5 μmol; magnesium acetate, 3 μmol; mercaptoethanol, 3 μmol; [γ-^{32}P] ATP (0.2 μCi), 2.5 nmol; histone, 50 μg; and protein kinase (about 5 μg of protein) with or without cyclic AMP, $2 \cdot 10^{-4}$ μmol. The incubation was carried out at 37° C for 5 min and reaction was stopped by the addition of 5 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate (pH 2.0), the precipitates were collected in a glass filter. After extensive washes with trichloroacetic acid-sodium tungstate, acetone, ethanol, and ether, the sample was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Protein concentration was determined by the method of Lowry et al. [18].

Results

Purification of protein kinase

All operations were done at 0—4° C unless otherwise stated. A 40 ml portion of the 105 000 × *g* supernatant solution (approx. 45 mg of protein) was brought to pH 4.5 by the careful addition of 1 M acetic acid with stirring. The precipitate obtained after centrifugation was dissolved in 4 ml of 0.05 M Tris · HCl buffer (pH 8.5) containing 0.05 M NaCl and 6 mM mercaptoethanol, and dialysed against the same buffer overnight. The resulting precipitate was discarded by centrifugation. The dialysed solution was applied to DEAE-Sephadex A-50 (1.0 cm × 16 cm), which had been equilibrated with 0.05 M Tris · HCl buffer (pH 8.5) containing 0.05 M NaCl and 6 mM mercaptoethanol buffer. Elution was carried out with a 120 ml linear concentration gradient of NaCl (0.05—0.6 M) in 0.05 M Tris · HCl buffer containing 6 mM mercaptoethanol, and 3 ml of fractions were collected. The enzyme activity was eluted as a single peak in the tubes No. 18—30, as shown in Fig. 1. The fraction of this peak and other fractions were not stimulated nor inhibited by adding the cyclic nucleotides. At the increasing concentration up to 10^{-5} M of cyclic AMP, protein kinase activity could not be stimulated, either. By using the purification procedure and the assay conditions described above, the specific activity of partially

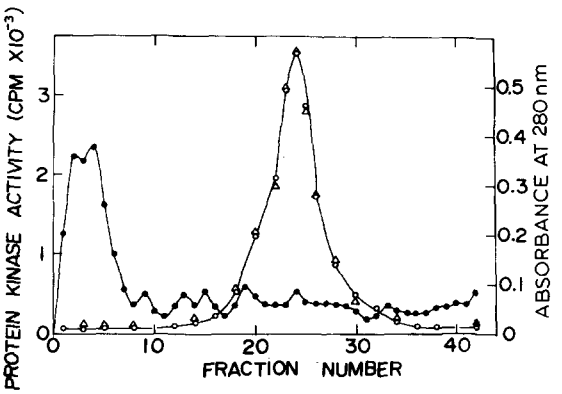


Fig. 1. DEAE-Sephadex column chromatography of protein kinase. The experimental conditions are described in the text. •, absorbance at 280 nm; ○, protein kinase activity; △, protein kinase activity with cyclic AMP ($8 \cdot 10^{-6}$ M).

purified enzyme was increased about 10-fold over that of the 105 000 × *g* supernatant. The partially purified protein kinase has no detectable activity of cyclic nucleotide phosphodiesterase.

Properties of purified protein kinase

The activities of both dialyzed 105 000 × *g* supernatant and partially purified protein kinase from cultured plant cells were assayed using histone as the substrate. Maximal activities of both enzymes in the presence or in the absence of cyclic AMP were obtained at a range of pH 7.5—9.0 and were not stimulated by the addition of cyclic AMP or cyclic GMP at both pH 7.5 and 9.0 (Table I). Crude protein kinase preparations and purified protein kinase do not bind any cyclic AMP.

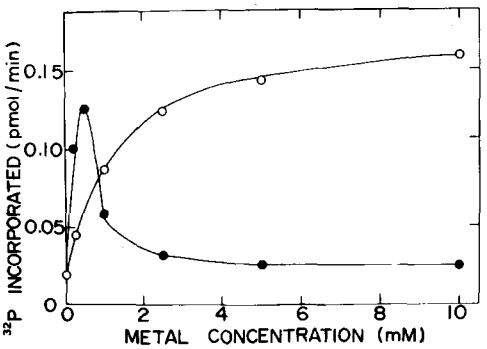
With partially purified enzyme, the effect of ATP and the substrate concentrations was studied. As calculated from the Lineweaver and Burk plot [19], the Michaelis-Menten constants (K_m) for ATP, histone, casein and prot-

TABLE I

EFFECT OF pH AND CYCLIC AMP ON HISTONE PHOSPHORYLATION

5 μg of partially purified protein kinase and 10 μg of crude preparation (dialized 105 000 × *g* supernatant) were used as enzymes. Assay conditions were employed as described in the text at the various pH values indicated in 0.05 M Tris · HCl buffer.

Enzymes	pH	Formation of acid insoluble ^{32}P (pmol/min)		
		Minus cyclic nucleotides	Plus cyclic AMP (10^{-6} M)	Plus cyclic GMP (10^{-6} M)
Crude preparations	7.5	0.02	0.02	0.02
	8.5	0.03	0.03	0.03
	9.0	0.02	0.02	0.02
Purified protein kinase	7.5	0.122	0.124	0.122
	8.5	0.150	0.149	0.152
	9.0	0.133	0.134	0.135



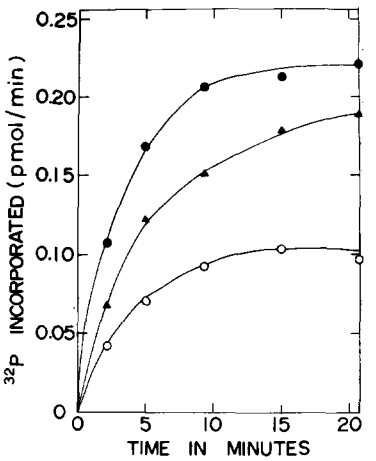


Fig. 2. The effect of varying concentration of Mg^{2+} or Mn^{2+} on the activity of protein kinase. Standard assay conditions are employed using various concentrations of Mg^{2+} (○) or Mn^{2+} (●) as indicated.

Fig. 3. Substrate specificity of protein kinase from cultured plant cells. Assay conditions are described in the text except for replacement of 200 μg/ml of histone (●) by 1200 μg/ml of casein (▲), or 200 μg/ml of protamine (○) as a substrate.

amine were approximately $7.1 \cdot 10^{-6}$ M, 50 μg/ml, 420 μg/ml and 66.7 μg/ml, respectively. V values for histone, casein and protamine were approximately 0.16, 0.12 and 0.08 pmol/5 min, respectively.

The protein kinase is completely dependent on the presence of a divalent metal for activity as shown in Fig. 2. The maximal activity by Mn^{2+} concentration showed a rather sharp peak at about 0.5 mM with activity, falling off very rapidly at higher levels (10 mM) of Mn^{2+}.

Fig. 3 shows the relative rates of reactions catalyzed by this protein kinase with histone, protamine and casein as substrates. The rate of phosphate incorporation at pH 8.5 is in the order histone > casein > protamine with relative activity rate of 1 : 0.8 : 0.4, taking the activity with histone as substrate to be 1, as shown in Fig. 3. Histones were also found to be more effective than protamine as substrates for the cyclic AMP-dependent protein kinases from liver [2] and brain [3].

As shown in Table II, the enzyme preparation possessed an activity of protein kinase which depended on the presence of histone as a substrate. In the absence of mercaptoethanol, *p*-chloromercuribenzoate inactivated the kinase activity, and this inactivation was overcome by adding mercaptoethanol, indicating that the enzyme has sulfhydryl groups.

The thermal stability of the protein kinase was determined by assaying the activity remaining after incubation for 5 min at various temperatures between 20—70°C in 0.05 M Tris · HCl buffer (pH 8.5). The results shown in Fig. 4 indicate that the activity of the enzyme was almost completely destroyed at temperatures higher than 45°C under the experimental conditions. The kinase activity is relatively stable at —20°C.

Not shown in the figure, the results were obtained that the ^{32}P-incorporated protein was hydrolyzed, the amino acids were separated by high-voltage

TABLE II

EFFECT OF SULFHYDRYL REAGENTS ON PROTEIN KINASE ACTIVITY

Assay conditions of protein kinase were described in the text.

Components	Formation of acid insoluble ^{32}P (pmol/min)
Complete system	0.168
– Histone	0.010
– Mercaptoethanol	0.145
– Mercaptoethanol + *p*-chloromercuribenzoate (0.5 mM)	0.030
+ *p*-chloromercuribenzoate (0.5 mM) + mercaptoethanol (20 mM)	0.160
+ cyclic AMP ($8 \cdot 10^{-7}$ M)	0.170
(10^{-5} M)	0.166
+ cyclic GMP ($8 \cdot 10^{-7}$ M)	0.172
(10^{-5} M)	0.168

electrophoresis and the radioactivity cochromatographed with authentic samples of phosphoserine and phosphothreonine.

Discussion

Ralph et al. [11] have reported the presence of protein kinase in Chinese-cabbage leaf, although Kuo and Greengard [10] failed to detect cyclic AMP-dependent protein kinase activity in plant tissues tested, and they showed that protein phosphorylation of isolated ribosome in vitro was not affected by cyclic AMP, indolyl-3-acetic acid or gibberellic acid.

To date there has been no evidence that cyclic AMP regulates the activity of a variety of specific protein kinases in plants although the presence of cyclic AMP in plant tissues was demonstrated [20—24]. Ralph et al. [11] suggested that the cytokinins control the phosphorylation of key proteins in plants; in this respect they play a similar role to cyclic AMP in animal cells.

Protein-kinase activity described in this paper was not stimulated by the addition of cyclic AMP even in crude extracts, as shown by Ralph et al. [11].

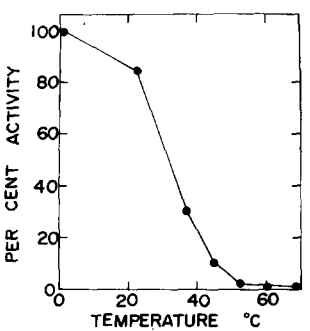


Fig. 4. Thermal stability of the partially purified protein kinase from plant cells. The activity of protein kinase was assayed after heating for 5 min in 0.05 M Tris · HCl buffer (pH 8.6) at the indicated temperatures. Assay conditions are described in the text.

Several protein kinases in a variety of animal tissues have recently been shown to consist of cyclic AMP-binding regulatory and independent catalytic subunit of the kinase molecule to activate the latter.

In 1974, Giannattasio et al. [13] reported the presence of cyclic AMP-binding protein in Jerusalem artichoke rhizome tissues. However, attempts to show that this protein is involved in the functioning of cyclic AMP-dependent protein kinase have failed.

The function of cyclic AMP in plant tissues still remains to be clarified.

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References

1 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3765
2 Langan, T.A. (1968) Science 162, 579—581
3 Jergil, B. and Dixon, G.H. (1970) J. Biol. Chem. 245, 425—434
4 Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395—6402
5 Corbin, J.D. and Krebs, E.G. (1969) Biochem. Biophys. Res. Commun. 36, 328—336
6 Brostrom, M.A., Reinmann, E.M., Walsh, E.A. and Krebs, E.G. (1970) Adv. Enzyme Regul. 8, 191—203
7 Gill, C.N. and Garren, L.D. (1970) Biochem. Biophys. Res. Commun. 36, 328—334
8 Jard, S. and Bastide, F. (1970) Biochem. Biophys. Res. Commun; 39, 559—566
9 Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 3417—3419
10 Kuo, J.F. and Greengard, P. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349—1355
11 Ralph, R.K., McCombs, P.J.A., Tener, G. and Wojicik, S.J. (1972) Biochem. J. 130, 901—911
12 Keates, R.A.B. (1973) Biochem. Biophys. Res. Commun. 54, 655—661
13 Giannattasio, M., Carratù, G. and Tucci, G.A. (1974) FEBS Lett. 49, 249—253
14 Nishi, A. and Sugano, N. (1970) Plant Cell Physiol. 11, 757—765
15 Murashige, T. and Skoog, F. (1970) Physiol. Plant. 15, 473—497
16 Walton, G.M. and Garren, L.D. (1970) Biochemistry 9, 4223—4229
17 Yamamura, H., Tadeda, M., Kumon, A. and Nishizuka, Y. (1970) Biochem. Biophys. Res. Commun. 40, 675—682
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Lineweaver, H. and Burk, O. (1934) J. Am. Chem. Soc. 56, 658—666
20 Raymond, P., Narayanan, A. and Pradet, A. (1973) Biochem. Biophys. Res. Commun. 53, 1115—1121
21 Brewin, N.J. and Northcote, D.H. (1973) J. Exp. Bot. 24, 881—888
22 Brown, E.G. and Newton, R.P. (1973) Phytochemistry 12, 2683—2687
23 Lunden, C.V., Wood, N.H. and Braun, A.C. (1973) Differentiation 1, 255—260
24 Giannattasio, M., Mandato, E. and Macchia, V. (1974) Biochem. Biophys. Res. Commun. 57, 365—371