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# CALCIUM-ACTIVATED PROTEIN KINASE FROM SOLUBLE AND MEMBRANE FRACTIONS OF MAIZE COLEOPTILES

N. H. Battey

Department of Horticulture, Plant Science Laboratories, University of Reading, Whiteknights, Reading, RG6 2AS, U.K.

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This paper describes the results of experiments in which phenyl Sepharose was used to partially purify $Ca^{2+}$-activated protein kinase (CPK) from maize soluble and membrane-solubilized proteins. It is shown that CPK has very similar properties to $Ca^{2+}$-activated, calmodulin independent protein kinase from other plant tissues, and that chromatography on phenyl Sepharose resolves two closely related forms of CPK from both soluble and membrane-solubilized proteins. The amount of each of these forms differs in the two fractions, and it is suggested that the kinase requiring EGTA for elution from phenyl Sepharose at high pH may be either a non-proteolitically digested form or an acylated form of CPK.

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There is great interest in protein kinases from plant tissues because of their potential importance in the regulation of plant metabolism. $Ca^{2+}$-regulated protein kinases are of special significance because of the key regulatory role that $Ca^{2+}$ plays in plant cells [1,2]. Over the past three years descriptions of a major $Ca^{2+}$-dependent protein kinase (CPK) activity from plants have appeared [3-7]. This protein kinase has been fully or partially purified from membrane or soluble fractions of a variety of tissues from different species (soluble fraction of soybean cell suspensions and alfalfa endosperm; membrane fraction of apple fruit), and has characteristics which include calmodulin independence, a $Ca^{2+}$-dependent mobility shift on SDS-PAGE, inhibition by low concentrations of calmodulin antagonists, binding to affinity matrices typically used for calmodulin purification (phenyl Sepharose, phenothiazine-linked Sepharose), and an $M_r$ of ~ 45 - 56,000 [3,7]. The function of this kinase activity is not known but its sensitivity to sub-$\mu$M levels of free $Ca^{2+}$ and its wide distribution suggest an important role in regulation in plants.

Previously it was established that chromatography on phenyl Sepharose could be used to resolve $Ca^{2+}$-dependent, calmodulin-independent protein kinase from $Ca^{2+}$-independent activity and from calmodulin (4,8). The experiments reported here aimed to extend these observations and compare $Ca^{2+}$-activated protein kinase from the soluble and from the membrane compartment of maize coleoptile tissue, looking specifically at the behaviour of the kinases from the two fractions on phenyl Sepharose chromatography and on SDS-PAGE and their sensitivity to $Ca^{2+}$. The results suggest that both fractions contain a mixture of two related forms of a CPK and that the balance of these forms differs in the soluble and membrane compartments.

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## METHODS

Plant material

Seeds of *Zea mays* (cv. Clipper) were soaked overnight in running water, sown in vermiculite, and grown for five days in darkness. The etiolated coleoptiles and enclosed leaf rolls were harvested, frozen in liquid $N_2$, and stored frozen at -18°C until required.

Chemicals

[$\gamma$-$^{32}$P]ATP (specific activity 3000 Ci.mmol$^{-1}$) was obtained from Amersham International plc (Amersham, Bucks. UK). Bovine calmodulin was from Calbiochem (Cambridge,UK), phenyl Sepharose CL-4B (ligand density 40 $\mu$mol.ml$^{-1}$ of gel), Sephacryl S200 and Sephadex G25 (Medium) were from Pharmacia LKB (Milton Keynes, Bucks, UK). All other chemicals were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK), BDH Chemicals Ltd. (Dagenham, UK) or Fisons Ltd. (Loughborough, UK).

Preparation of membrane and soluble fractions in the presence of EDTA or $Ca^{2+}$

Maize coleoptiles were homogenized in a mortar and pestle with 2.5 volumes of 0.25M sucrose, 50 mM Tris-Acetate, pH8.0, 0.1mM $MgCl_2$, 1mM DTT, 0.5mM PMSF and 1mM EDTA (extraction buffer) or 1mM $CaCl_2$, strained through two layers of muslin and centrifuged at 3,300 xg(av) for 20 mins. The supernatant was centrifuged at 36,900 xg(av) for 45 mins. The pellets (membrane fration) were resuspended in 10 mM HEPES (pH7.4) and used for protein kinase assays. The supernatants (soluble fraction) were brought to 20% saturation with ammonium sulphate, centrifuged (giving no visible pellet), and then brought to 80% saturation and re-centrifuged (36,900 xg(av) for 10 min). The pellets were dissolved in 10mM HEPES (pH7.4), and de-salted on a 5.5 x 1.5 cm column of Sephadex G25 into the same buffer, before assaying for protein kinase.

Solubilisation of membrane protein with EGTA

Maize coleoptiles were extracted as described above except that 2 volumes of extraction buffer containing EDTA were used, and 1M $CaCl_2$ was added to the 3,300 xg supernatant to give a free $Ca^{2+}$ concentration of approx. 1 mM before centrifugation at 30,000 xg (av) for 45 mins. The pellets were resuspended in 0.25 M sucrose, 50mM Tris-Acetate (pH 8.0), 10mM $MgCl_2$, 1mM $CaCl_2$, 0.5 mM EGTA, 1mM DTT, 0.5mM PMSF, and 25 $\mu$M ADP. After 5 min at room temperature, 100 mM EGTA was added to a final concentration of 6mM. The samples were then centrifuged at 30,000 xg (av) for 45 min, the pellets resuspended in extraction buffer and the supernatant de-salted into extraction buffer. The samples were then assayed for protein kinase activity.

Chromatography on phenyl Sepharose

This was carried out as described in detail by Battey and Venis [8] for soluble extracts. Membrane proteins solubilised with EGTA were prepared as described above, except that after the first 30,000 xg (av) centrifugation, the pellets were resuspended in extraction buffer containing 5mM EGTA, left for 15 min on ice, and centrifuged at 30,000 xg (av) for 45 mins. The supernatant was then made 10 mM in $CaCl_2$, centrifuged at 30,000 xg (av) for 10 mins, and loaded on the phenyl Sepharose column.

Protein kinase assays and gel electrophoresis

Assays for protein kinase and SDS-PAGE were carried out as described [8]. Free $Ca^{2+}$ levels in the protein kinase assays were set according to Robertson and Potter [9]. For detection of phosphorylated proteins on SDS-PAGE, samples were incubated for 2 minutes at room temperature in a buffer containing 2.2 $\mu$Ci[$\gamma$-$^{32}$P] ATP (carrier-free), 10mM $MgCl_2$, 50mM HEPES (pH 7.0), 0.9mM EGTA, and approx. 1.5 mM $CaCl_2$. Labelled proteins were precipitated with TCA and prepared for electrophoresis as described previously [8].

## RESULTS

Both the soluble and membrane fractions of maize coleoptiles contain CPK. Extraction of the tissue in EDTA gave better recovery of activity than extraction in $CaCl_2$, both in the membrane and the soluble fractions (Table 1). In both extractions membranes contained just over twice as much total kinase activity as the soluble fraction, and $Ca^{2+}$-activation varied from 1.5 - 3-fold. There was no evidence for $Ca^{2+}$-dependent binding of kinase to membranes (data not shown). However, 20 - 25% of the membrane protein kinase can be eluted from the membranes

TABLE 1

Protein kinase activity from the membrane and soluble fractions of maize

| Extraction with | Total activity (pmol Pi $sec^{-1}$.g $fwt^{-1}$) | Specific Activity (pmol Pi $sec^{-1}$.$mg^{-1}$) |
|---|---|---|
| EDTA | 3.5/1.5 | 1.9/0.6 |
| $Ca^{2+}$ | 2.1/0.9 | 2.1/0.5 |

Fractions were assayed for protein kinase in the presence of 300 μM free $Ca^{2+}$ after extraction of maize coleoptiles in buffer containing 1mM $CaCl_2$ or 1mM EDTA. Left figure - membrane activity/ right figure - soluble activity.

with EGTA (Table 2). Similar amounts of activity were solubilized by EGTA regardless of whether an ATP or ADP pre-treatment, or no pre-treatment at all was given (data not shown).

The CPK in the soluble fraction, and that solubilised by EGTA were analysed by chromatography on phenyl Sepharose. As previously reported [4,8] most $Ca^{2+}$-activated protein kinase can be eluted by raising the pH to 8.5 (in the presence of $Ca^{2+}$), and then washing with EGTA. However, the proportion of kinase eluting as the pH was increased, rather than on subsequent EGTA-washing, was typically much greater in the soluble extract than in the solubilised extract (Table 3). When the activity is calculated per mg of total membrane or soluble protein, it is clear that Fraction B contains a relatively constant amount of activity, whereas Fraction A is reduced by about 90% in the membrane-solubilised extracts. Therefore the difference is a consequence of the much lower levels of kinase activity in Fraction A from membrane-solubilised extracts.

When samples from Fractions A and B were labelled with [$\gamma$-$^{32}$P]ATP, subjected to SDS-PAGE and autoradiographed, the labelled proteins were of $M_R$ 51-58000 (Figure 1). This approximates to the $M_R$ of the autophosphorylated CPK from soybean, apple and alfalfa (between 45 and 56000) [3,5,6]. The phosphorylated proteins also showed the $Ca^{2+}$-dependent mobility shift characteristic of CPK from other plants (Figure 1), and so probably represent autophosphorylated CPK from maize. This autophosphorylated kinase from Fraction B had a slightly higher $M_R$ in both soluble and membrane-solubilised samples, than that from Fraction A (Figure 1).

TABLE 2

Elution of CPK from maize microsomal membranes with EGTA. Membranes were resuspended in buffer before homogenising with 6mM EGTA and recentrifugation. The supernatants were de-salted before assay for protein kinase

| Fraction | Total activity (pmol Pi $sec^{-1}$ .g $fwt^{-1}$) | Specific activity (pmol Pi $sec^{-1}$. $mg^{-1}$) | $Ca^{2+}$-activation[1] |
|---|---|---|---|
| Total membranes | 2.4 | 4.3 | 2.5 |
| EGTA-treated: membrane bound | 1.3 | 2.0 | 2.4 |
| solubilised | 0.4 | 3.7 | 4.9 |

[1] Activity in the presence of μM free $Ca^{2+}$/activity in the presence of excess EGTA.

TABLE 3

Resolution of $Ca^{2+}$-activated protein kinase on phenyl Sepharose. Samples were loaded in the presence of $Ca^{2+}$, and the column eluted with salt, and EGTA (pH 6.8) to remove calmodulin (Ref. 8), before elution by increasing pH (6.8 to 8.5, + $Ca^{2+}$) and then EGTA (pH 8.5) (see Methods)

| | Column elution | | | | |
|---|---|---|---|---|---|
| | Fraction A [pH 6.8 to 8.5 (+$Ca^{2+}$)] | | Fraction B [EGTA (pH 8.5)] | | % in EGTA (pH 8.5) |
| | Total activity[1] | Specific activity[2] | Total activity[1] | Specific activity[2] | |
| Soluble | 2.7 | 3.5 (~0.24 mg$^{-1}$soluble protein)+ | 0.7 | 39.0 (~0.07 mg$^{-1}$soluble protein)+ | 21 |
| Membrane solubilised By EGTA | 0.07 | 4.4 (~0.04 mg$^{-1}$membrane protein)+ | 0.06 | 62.6 (~0.04 mg$^{-1}$membrane protein)+ | 46 |

[1] pmol Pi sec$^{-1}$ gfwt$^{-1}$

[2] pmol Pi sec$^{-1}$ mg$^{-1}$

+ These figures express the amount of activity in the fraction per mg of crude membrane or soluble protein, and, assuming no losses during purification, should reflect the original specific activity of the enzyme that elutes in each fraction.

When the pCa optima of CPK in the two post-phenyl Sepharose fractions were compared, both forms of the enzyme showed maximum activation at a pCa of approx. 5 (Figure 2). $Ca^{2+}$-activation is rather variable but does not appear to differ consistently in the different fractions. Calmodulin at 1μM did not stimulate CPK from either fraction at any pCa value (data not shown).

## DISCUSSION

The evidence presented in this paper suggests that maize contains a $Ca^{2+}$-activated protein kinase of similar properties to those purified from soybean,

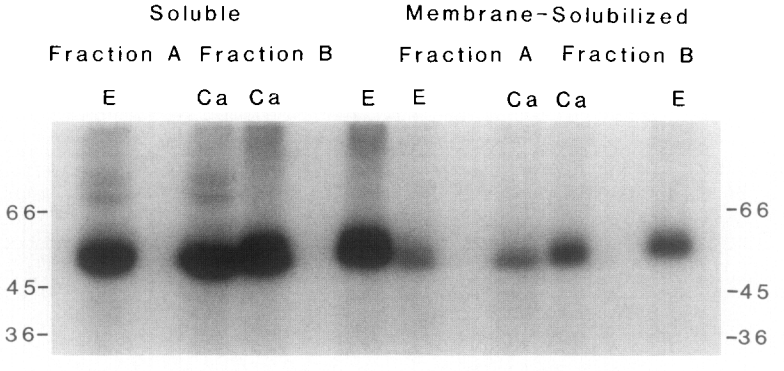


Figure 1. Autoradiograph showing $Ca^{2+}$-dependent, calmodulin independent protein kinase from maize. Soluble and membrane-solubilized proteins from maize coleoptiles were chromatographed on phenyl Sepharose and eluted by raising the pH (Fraction A) and then washing with EGTA (Fraction B) (see 'Methods' for detail). Samples of each fraction were labelled with [$\gamma^{32}$P] ATP, TCA precipitated and subjected to 12% SDS-PAGE in the presence of 1mM EGTA (E) or 1mM $Ca^{2+}$ (Ca). $M_R$(x $10^{-3}$) of molecular weight standards are indicated.

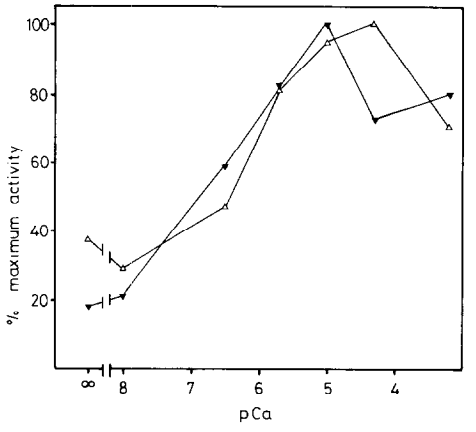


Figure 2. pCa optima of CPK from different sources and post-phenyl Sepharose fractions. Protein from the soluble fraction (△), or membrane-solubilized using EGTA (▼), was chromatographed on phenyl Sepharose and eluted by raising the pH (△) and then washing with EGTA (▼).

alfalfa and apple. Furthermore, it shows that the enzyme is present in both the soluble and the membrane compartment, but that two different forms, resolved by chromatography on phenyl Sepharose, exist in both compartments. The form demonstrating $Ca^{2+}$-dependent hydrophobic binding at high pH is relatively more abundant in the membrane-solubilised material.

Polya and co-workers have described two forms of $Ca^{2+}$-dependent protein kinase from silver beet leaves [10,11]. These two enzymes can be resolved on DEAE-Sephacel; interestingly, the balance of types I and II is different between the soluble and membrane compartments [12]. The native molecular size is between 50 and 60000.

The CPK eluting in Fraction B on phenyl Sepharose could be a covalently modified form of the enzyme from Fraction A. Acylation is known to increase the hydrophobicity, and hence membrane-binding, of some tyrosine kinases as well as other proteins [see 12, 13 for reviews]. Alternatively the enzyme in Fraction A may be a partially proteolysed, less hydrophobic form of that in Fraction B. Work to distinguish between these two possibilities is now a priority. The fact that the membrane enzyme can be solubilised with EGTA suggests that $Ca^{2+}$ could regulate binding of the enzyme to membranes although a similar amount of CPK can be solubilised by acetone precipitation (data not shown). It remains to be seen whether $Ca^{2+}$-dependent membrane association, of the type exhibited by protein kinase C [14, 15], or by other groups of proteins such as the annexins [16, 17], is an important feature of plant CPK.

The CPK described here and in other plant tissues appears distinct from $Ca^{2+}$/calmodulin-dependent protein kinase II from animals, although the autophosphorylatable sub-units of the latter have $M_R$ values between 50 and 60,000 [18, 19]. It is also distinct from protein kinase C. It is now important to discover the relationship between plant CPK and other kinases, the function of the membrane and soluble forms of CPK in plant tissues and to establish whether the distribution between membranes and cytoplasm is regulated by intracellular $Ca^{2+}$ levels.

## REFERENCES


1. Trewavas, A.J. (1986) (ed.) *Molecular and cellular aspects of calcium in plant development.* NATO ASI series, Vol. 104. Plenum Press, New York, London.

2. Allan, E. and Trewavas, A.J. (1987) The role of calcium in metabolic control. In: *The Biochemistry of Plants Vol. 12,* pp. 117-149. Academic Press.

3. Harmon, A.C., Putnam-Evans, C. and Cormier, M.J. (1987) *Plant Physiology* **83**, 830-837.

4. Battey, N.H. and Venis, M.A. (1988) *Anal. Biochem.* **170**, 116-122.

5. Battey, N.H. and Venis, M.A. (1988) *Planta* **176**, 91-97.

6. Bögre, L., Olah, Z. and Dudits, D. (1988) *Plant Science* **58**, 135-144.

7. Olah, Z., Bögre, L., Lehel, C., Farago, A., Seprodi, J. and Dudits, D. (1989) *Plant Molecular Biology* **12**, 453-461.

8. Battey, N.H. and Venis, M.A. (1990) Calcium-dependent hydrophobic interaction chromatography. In: *Practical Protein Chromatography (Methods in Molecular Biology)* (eds. A. Kenney and S. Fowell). Humana Press, U.S.A. (in press)

9. Robertson, S. and Potter, J.D. (1984) *Methods Pharmacol.* **5**, 63-75.

10. Polya, G.M., Klucis, E. and Haritou, M. (1987) *Biochim. Biophys. Acta* **931**, 68-77.

11. Klucis, E. and Polya, G.M. (1988) *Plant Physiol.* **88**, 164-171.

12. Magee, T. and Hanley, M. (1988) *Nature* **335**, 114-115.

13. Sefton, B.M. and Buss, J.E. (1987) *J.Cell Biol.* **104**, 1449-1453.

14. Wolf, M., Cuatrecasas, P. and Sahyoun, N. (1985) *J.Biol.Chem.* **260**, 15718-15722.

15. Ito, T., Tanaka, T., Yoshida, T., Onoda, K., Ohta, H., Hagiwara, M., Itoh, Y., Ogura, M., Saito, H. and Hidaka, H. (1988). *J.Cell Biol.* **107**, 929-937.

16. Geisow, M.J. and Walker, J.H. (1986) *Trends Biochem.Sci.* **11**, 420-423.

17. Drust, D.S. and Creutz, C.E. (1988) *Nature* **331**, 88-91.

18. McGuiness, T.L., Lai, Y., Greengard, P., Woodgett, J.R. and Cohen, P. (1983) *FEBS Lett.* **163**, 329-334.

19. McGuiness, T.L., Lai, Y. and Greengard, P. (1985) *J.Biol.Chem.* **260**, 1696-1704.