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PARTIAL PURIFICATION AND PROPERTIES OF A PROTEIN KINASE C TYPE ENZYME FROM PLANTS

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Key Word Index—*Amaranthus tricolor*; Amaranthaceae; protein kinase C; calcium-dependence; phospholipid-activation.

Abstract—Partial purification of a protein kinase with a dependence on micromolar concentrations of free calcium has been achieved from seedlings of *Amaranthus tricolor*. The enzyme has a M_r of 77 600 as determined by gel filtration and 84 500 by SDS–PAGE analysis. Interaction of the enzyme with membranes (inside-out erythrocyte vesicles) is regulated by calcium, a characteristic of animal protein kinase C. Phospholipid and diolein activation of the enzyme is markedly dependent on the phospholipid used and on both calcium and phospholipid concentration. K_m values for Ca^{2+} in the absence of phospholipid was 20–40 μM and in the presence of phosphatidylserine 5–10 μM. Diolein plus phosphatidylserine lowered the K_m to < 1.5 μM. The best activation was achieved at 100 μM calcium with 40 μg/ml phosphatidylserine and 8 μg/ml diolein. These properties indicate a protein kinase C type enzyme. The plant enzyme reacted with antiserum directed against the regulatory domain of bovine brain protein kinase C in an immunoblot experiment.

INTRODUCTION

There is now strong evidence (reviewed in [1, 2]) that in plants calcium is a second messenger in transmembrane signalling by hormones, light and other environmental stimuli. There are many instances of physiological responses to such stimuli being modified by added calcium, by inhibitors of calcium transport or by calcium ionophores. Some components of the calcium signal system as found in animals also occur in plants [3]. The Ca^{2+}-dependent regulatory protein, calmodulin, has been shown to occur in plants, with many properties strikingly similar to that from animals. Several plant enzymes are known to be activated by the Ca^{2+}-calmodulin complex; these include NAD kinase, Ca^{2+}-transport ATPase, quinate: NAD^+ oxidoreductase, soluble and membrane-bound protein kinases and H^+-transport ATPase [3].

In animals, signal transduction involves diacylglycerol as well as calcium, the two acting synergistically [4]. The diacylglycerol arises from hydrolysis of polyphosphoinositides. In plants a number of studies have now identified phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate [5–8] and shown the presence of phosphatidylinositol phospholipase C which cleaves phosphoinositides between phosphate and glycerol [9, 10]. Auxin has been found to stimulate the hydrolysis of phosphatidylinositol in isolated soybean membranes [11] and there is a report of diacylglycerol stimulation of phosphorylation in isolated soybean membranes [12]. Diacylglycerol and calcium have been shown to act synergistically in the cytokinin-dependent betacyanin response in *Amaranthus tricolor* seedlings [2]. This synergism is characteristic of the dual signal pathway in the animal hormone model [4].

The result of diacylglycerol release in animal cell membranes is to activate protein kinase C leading to phosphorylation of a particular set of proteins in the cell. Together with phosphorylation of another set by calcium–calmodulin-depedent protein kinase, this results in the subsequent cellular response [4]. The major gap in the evidence for a signal transducing system in plants analogous to that described for animals has been the absence of compelling evidence that protein kinase C exists in plants. In this laboratory [13] it was shown that a calcium- and phospholipid-dependent protein kinase exists in *A. tricolor* seedlings and soybean callus tissue. Schäfer et al [14] also showed a calcium-, phospholipid-kinase activity in zucchini hypocotyls [14]. More recently [15] we showed that the enzyme from *A. tricolor* cross-reacts with an antibody raised against the amino acid sequence 280 to 292 of bovine brain portein kinase C [16].

In this paper we describe the partial purification and properties of the *A. tricolor* kinase. The results suggest that protein kinase C probably plays a pivotal role in plants as it does in animals.

RESULTS

A calcium-dependent, phospholipid-activated protein kinase was purified 460-fold (Table 1) from a 100 000 g supernant from extracts of *A. tricolor* half-seedlings. The final preparation had a specific activity of $\sim$ 3.4 nmol/min/mg protein, measured in the presence of 5 μM ATP. The chromatographic steps in the purification were anion exchange (DEAE-Sephacel, Fig. 1A), affinity chromatography (Phenyl-Sepharose CL-4B, Fig. 1B) and gel filtration (Sephacryl S-200, Fig. 1C). Size exclusion on the Sephacryl S-200 indicated a protein of M_r 77 600.

Although DEAE-Sephacel adsorption brought about a 15-fold purification it did not remove a protease which was detected as a histone hydrolysing activity during long

Table 1. Partial purification of protein kinase

Purification step	Protein (mg)	Protein Kinase (pmol/min/mg protein)	Purification
Filtered supernatant	124.4	7.3	
DEAE-Sephacel	58.3	108.4	15
Phenyl-Sepharose CL-4B	9.4	651.4	89
Sephacryl S-200	9.1	3357.8	460

30 g *A. tricolor* half-seedlings were the starting material. Protein kinase was assayed in the standard manner with the following calcium conditions. Filtered supernatant: 0.5 mM EDTA, 1.25 mM EGTA, 5 mM Ca^{2+} (3.26 mM free Ca^{2+}); DEAE-Sephacel: 0.5 mM EDTA, 1.25 mM EGTA, 4.0 mM Ca^{2+} (2.27 mM free Ca^{2+}); Phenyl-Sepharose CL-4B: 0.125 mM EDTA, 0.125 mM EGTA, 1.2 mM Ca^{2+} (1 mM free Ca^{2+}); Sephacryl S-200: 0.5 mM EDTA, 1.25 mM EGTA, 1.6 mM Ca^{2+} (100 μM free Ca^{2+}).

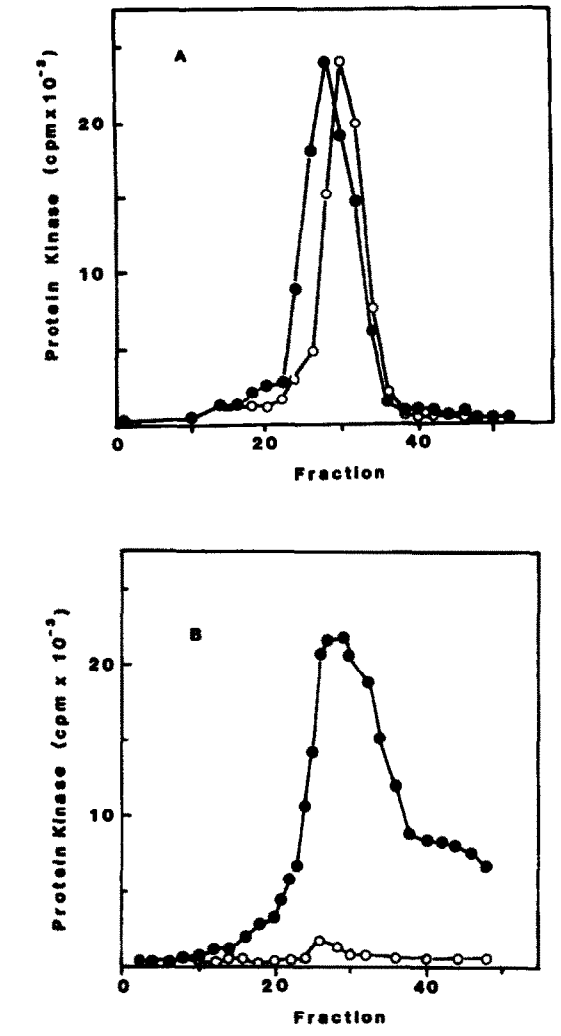
term (60') protein kinase assays and was found to co-purify with the protein kinase [17]. The addition of the protease inhibtor, leupeptin, to fractions from the DEAE-Sephacel protected the protein kinase from proteolysis. Phenyl-Sepharose chromatography separated the protease from the protein kinase activity. The final preparation from the Sephacryl column was stable at 4° for at least four weeks. Phenyl-Sepharose also removed the calcium-independent kinase activity which was only partially separated on the DEAE-Sephacel column, being eluted at a higher salt concentration than the calcium, phospholipid-dependent enzyme.

In other purification experiments not shown in Table 1 an attempt was made to use an affinity column of phosphatidylserine immobilised in polyacrylamide [18]. In three of five experiments binding of calcium-dependent protein kinase to this column in 1 mM Ca^{2+} and elution by EDTA was achieved but the enzyme appeared to be unstable following this treatment. Another technique which has been developed with success in animal protein kinase C purification is the use of calcium-dependent binding to inside-out erythrocyte membrane vesicles [19] as a preliminary step. Using the same conditions of binding to these membranes as used for rat brain extracts we have observed binding of about 50% of the plant enzyme. The reason for incomplete binding is not known. Release of the bound plant protein kinase from the vesicles in the presence of EDTA and EGTA was achieved as for rat protein kinase C [19].

SDS-PAGE analysis of the fractions from a number of purification steps is shown in Fig. 2. Peak fractions from the DEAE-Sephacel separation shown in Fig. 1D contain several proteins stained by Coomassie Blue which appear to autophosphorylate in a calcium-dependent manner (Fig. 2A) and which correlate with the activity through the major protein kinase peak. Among these proteins, those with M_r 94000, 84000, 67000 and 34500 are most apparent. In addition there is phosphorylation of species at M_r 58500 and 51000 which are partly calcium-dependent in fractions 26–30 but not in later fractions (32–40; data for 34–40 not shown). In Fig. 2B a highly concentrated preparation from pooled fractions 46–55 of the Sephacryl S-200 fractionation shown in Fig. 1C has been used to demonstrate the SDS-PAGE/ autophosphorylation pattern of the final partially purified enzyme. The phosphorylation of bands in this preparation include most of those noted in Fig. 2A, with the addition of a number of lower M_r species, possibly proteolysis products. Further analysis of this preparation by the Western blot technique was undertaken [15] and the conclusions are summarized in the discussion. In Fig. 2C is shown the SDS-PAGE/autophosphorylation analysis of a preparation that was given a preliminary purification by calcium-dependent binding onto inside-out erythrocyte membrane vesicles [19] followed by elution in EDTA and EGTA and then DEAE-Sephacel fractionation. While it is not clear by comparison with Fig. 2A that the vesicle step has removed some contaminating protein the purification achieved to this stage was 50-fold compared to 15-fold at the equivalent stage in Table 1.

During the purification the optimum Ca^{2+} concentration decreased from 3.26 mM for the crude extract to 2.27 mM for the enzyme from DEAE-Sephacel, to 1 mM from Phenyl-Sepharose and to 100 μM for the enzyme from Sephacryl. This may be due to removal of other calcium-binding proteins during purification. The need for a strongly calcium buffered system when making these measurements is seen in Fig. 3 where a higher K_m for Ca^{2+} is seen in a lightly buffered incubation mixture (using 0.25 mM EDTA/EGTA) than in a heavily buffered incubation mixture (using 1.43 mM EDTA/EGTA). Presumably, with less buffering capacity, calcium binding proteins alter the free calcium concentration. (Care needs to be taken with pH adjustment when high Ca^{2+} buffering is used because of the release of protons when Ca^{2+} and EGTA interact). The effect of various phospholipids and of diolein on the activity of the protein kinase are shown in Figs 4 and 5. In the absence of diolein (Fig. 4) phosphatidylserine and phosphatidylethanolamine (both at 8 μg/ml) are equally effective. However only in the phosphatidylserine is there an added effect by diolein, the Ca^{2+} K_m being lowered from 8 to < 1.5 mM by the diacylglycerol (Fig. 5). This effect was best seen at 40 μg/ml phosphatidylserine and 8 μg/ml diolein.

The previously reported [13] preference of this enzyme for histone as a substrate (compared with casein) was confirmed in an experiment that showed histone phosphorylation also had a much lower requirement for calcium (Fig. 6).



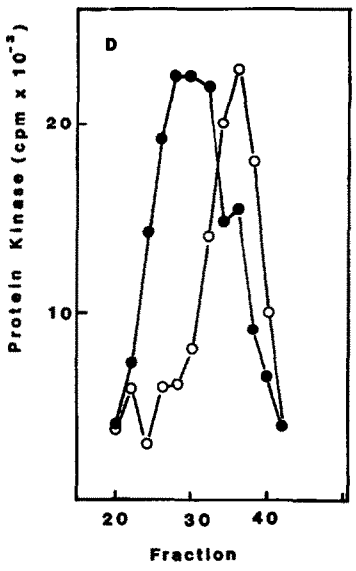


Fig. 1. Purification of protein kinase from 100,000 g supernatant of *A. tricolor* seedling extract. A, DEAE-Sephacel column fractionation; B, Phenyl-Sepharose CL-4B column fractionation of peak fractions 16–36 from A; C, Sephacryl S-200 column fractionation of peak fractions 10–60 from B; D, Part of a DEAE-Sephacel column fractionation supplying fractions, 28, 30 and 32 for SDS-PAGE analysis in Fig. 2A. Protein kinase activity was determined in the presence (●—●) or absence (○—○) of Ca^{2+} and PS as described in Experimental. Ca^{2+} conditions were as follows: DEAE-Sephacel: 0.5 mM EDTA, 1.25 mM EGTA, 5 mM Ca^{2+} (3.26 mM free Ca^{2+}); Phenyl-Sepharose CL-4B: 0.125 mM EDTA, 0.125 mM EGTA, 1.2 mM Ca^{2+} (1mM free Ca^{2+}); Sephacryl S-200: 0.5 M EDTA, 1.25 mM EGTA, 1.6 mM Ca^{2+} (100 μM free Ca^{2+}).

DISCUSSION

Activation of animal protein kinase C takes place either by partial proteolysis or by interaction with various lipids (see Review [20]). Proteolysis yields an M_r 51 000 enzyme which no longer needs phospholipid and calcium for activation. Such a proteolysis may be taking place in plant extracts in spite of the presence of leupeptin. The fractionation of *A. tricolor* extract from DEAE-Sephacel shows a large peak of calcium-independent kinase activity eluting from the column at a higher salt concentration than the calcium, phospholipid-dependent enzyme (Fig. 1A). This is similar to the behaviour on DEAE of animal protein kinase C in its calcium-dependent and calcium-independent forms. The autophosphorylated proteins in the early fractions of a DEAE-Sephacel column (Fig. 1A, fractions 26–30, coinciding with the calcium, phospholipid-dependent activity peak) were in the higher M_r range. Phosphorylated protein at M_r 94 000 is seen from fraction 24 to fraction 36, that at M_r 84 000 from fraction 30 to 40 and that at M_r 67 000 from fraction 26–32. On the other hand lower M_r species which were phosphorylated (M_r 58 500 and 51 000), while beginning to appear in the early fractions, mostly coincided with the calcium-phospholipid-independent peak. Both these species could still be seen up to fraction 44 (data for later fractions not shown).

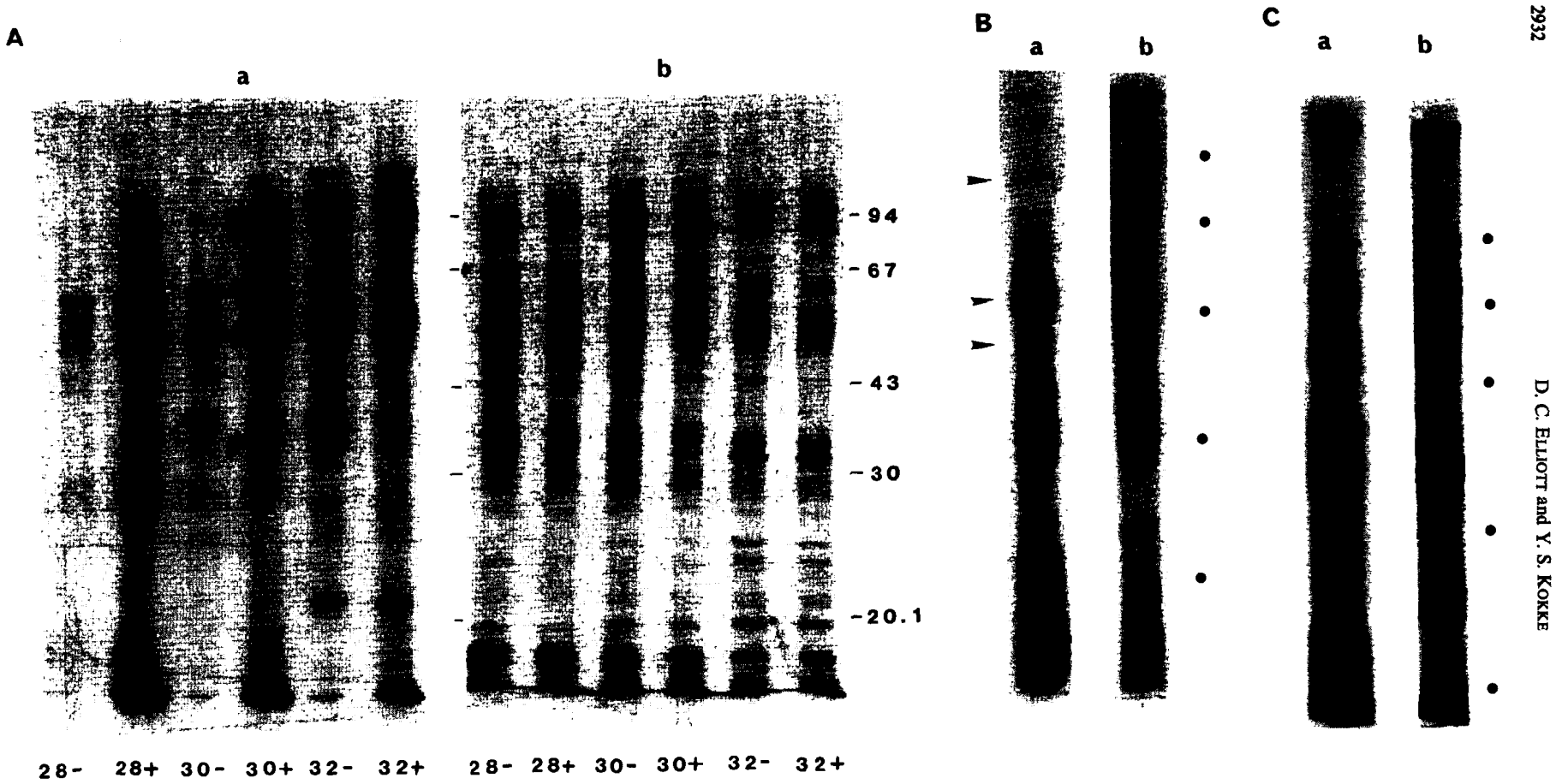


Fig. 2. Polypeptide composition and autophosphorylation of protein kinase preparations at different stages of purification. In each case *a* denotes autoradiography while *b* shows the Coomassie Blue stain. (A) SDS-PAGE analysis of fractions through the major Ca^{2+}/PS dependent protein kinase peak in Fig. 1D (28, 30, 32). Samples were incubated with [γ-^{32}P]ATP ± calcium phosphatidylserine and prepared as described in Experimental. (In the figure, fraction numbers are followed by − or + to denote − and + Ca^{2+}/PS). Approximately 15 μg protein was loaded per gel track. Stars indicate proteins which autophosphorylate in fraction 30 (M_r 94 00, 84 000, 58 500, 51 000, 34 500). (B). Autophosphorylation of peak fraction from gel filtration, the most highly purified enzyme. Fractions 46—55 from separation in Fig. 1C were pooled and concentrated using an Amicon ultra-filtration unit (PM 30 membrane), and incubated with [γ^{32}-P]ATP as described in Experimental. Approximately 20 μg protein was loaded for SDS-PAGE. Molecular size markers (Pharmacia LMW kit) indicated by dots were phosphorylase b (94 000), bovine serum albumin (67 000), ovalabumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100). Arrows indicate the position of proteins of M_r 84 500, 50 000 and 40 000, tentatively identified as native protein kinase C, the catalytic domain and the regulatory domain respectively (see Discussion), (C). Autophosphorylation of pooled and concentrated peak fractions from DEAE-Sephacel column following preliminary purification by calcium-dependent binding to inside-out erythrocyte vesicles. Molecular size markers as in Fig. 2B. Approximately 170 μg loaded.

Activation of animal protein kinase C by phospholipid is markedly specific [20] but with differences depending on the enzyme source. Phosphatidylserine is usually the most effective; phosphatidylethanolamine activates enzymes from some sources, while phosphatidylcholine is not an activator. The results with phospholipid and diolein (Figs 4 and 5) show that the plant enzyme has properties similar to those of protein kinase C. Phosphatidyserine and phsophatidylethanolamine were equally effective while phsophatidylcholine stimulated slightly. The calcium K_m with phosphatidylserine and phosphatidylethanolamine was the same (8–10 μM). Only the K_m in the presence of phosphatidylserine was altered by diolein, being reduced to < 1.5 μM. Without phospholipid the K_m was 20–40 μM.

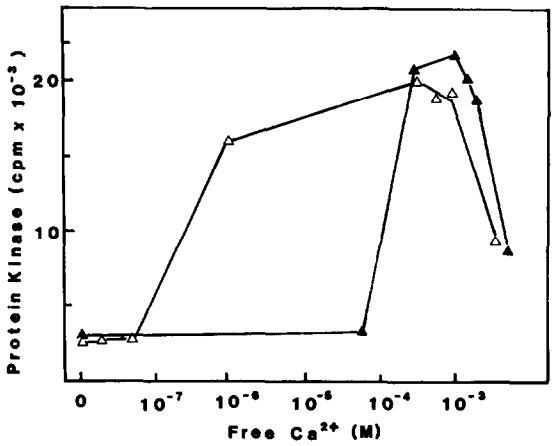


Fig. 3. Protein kinase activity in the peak fraction from a Phenyl-Sepharose column (similar to Fig. 1B fractionation). The free Ca^{2+} concentration was calculated for assay conditions using high (△——△) and low (▲——▲) calcium buffering capacity. High capacity mixtures contained 0.125 mM EDTA and 1.305 mM EGTA, while low capacity mixtures contained 0.125 mM EDTA and 0.125 mM EGTA.

Physiological regulation of animal protein kinase C requires its interaction with cellular membranes. Activation of the enzyme by diacylglycerol or tumour promoters results in translocation of the enzyme from a soluble to a membrane-bound compartment a process regulated *in vivo* by Ca^{2+} mobilisation and increase in intracellular Ca^{2+} [19]. The ability of inside-out erythrocyte vesicles to bind protein kinase C in the presence of calcium and reversal by calcium chelators has been used as a model to study this intracellular translocation. Our results show similar calcium-dependent enzyme-membrane association for the plant protein kinase.

In view of the similarity in these properties of the plant enzyme to those of animal protein kinase C (lipid activation and Ca^{2+}-dependent membrane association) a study was undertaken [15] to determine whether polyclonal antibodies raised against an amino acid sequence derived from bovine brain protein kinase C cross react with the plant enzyme. This peptide is part of the sequence which binds diacylglycerol and phorbol esters and is known to be part of the regulatory domain of the protein

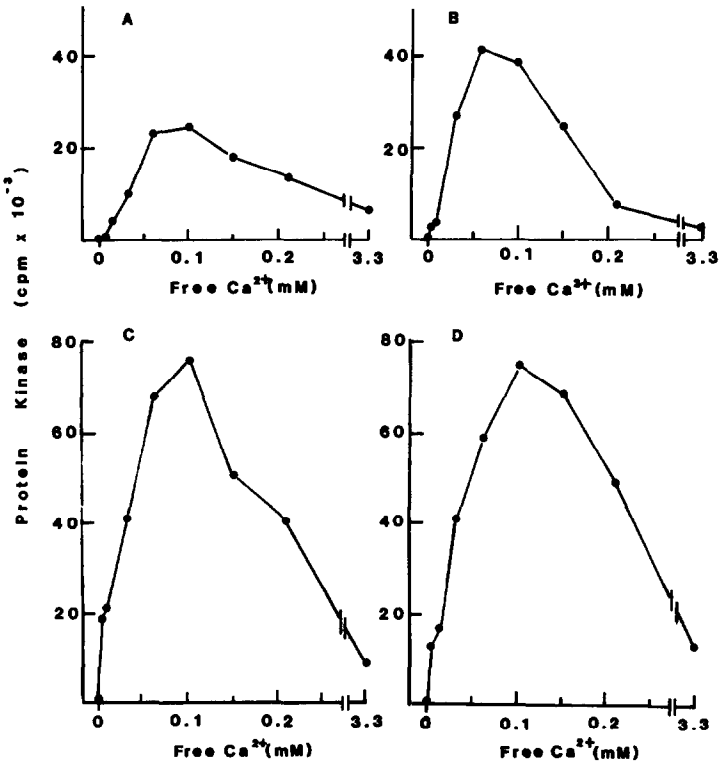


Fig. 4. Effect of phospholipids (8 μg/ml) at different calcium concentrations on protein kinase activity in the pooled peak fractions from a Sephacryl S-200 column (similar to Fig. 1C fractionation). A, no phospholipid; B, phosphatidylcholine; C, phosphatidylethanolamine; D, phosphatidylserine.

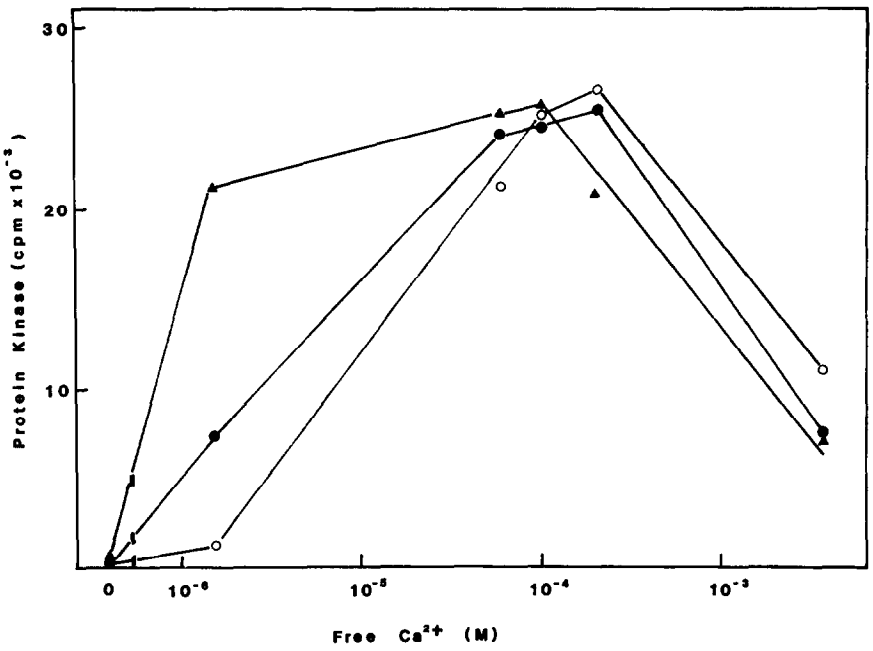


Fig. 5. Effect of diolein and phosphatidylserine on calcium dependence of protein kinase activity in the peak fraction from a Sephacryl S-200 column (similar to Fig. 1C fractionation). No additions (○——○); phosphatidylserine (40 μg/ml) alone (●——●); phsophatidylserine (40 μg/ml) plus diolein (8 μg/ml) (▲——▲).

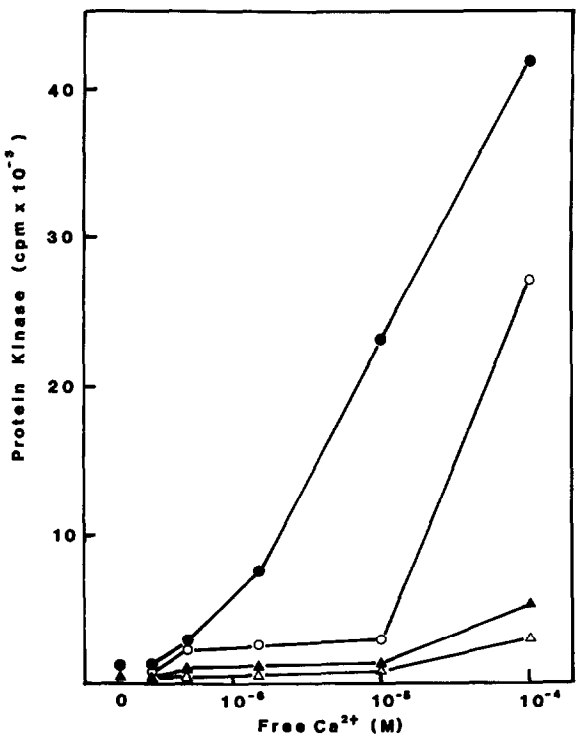


Fig. 6. Comparison of protein kinase substrates. The pooled peak fractions 46–55 from the Sephacryl S-200 column shown in Fig. 1C were used. Histone (167 μg/ml) without (○——○) or with (●——●) phosphatidylserine (40 μg/ml); casein (167 μg/ml) without (△——△) or with (▲——▲) phosphatidylserine (40 μg/ml).

[16]. Cross-reactivity of three major protein species of M_r 84 500, 65 000 and 40 000 was shown [15]. Comparison made with the ^{32}P-labelled species in the same preparation subjected to autophosphorylation (Fig. 2B) shows that at least two cross-reacting species were phosphorylated (M_r 84 500 and 40 000). By comparison with animal protein kinase C, these may be native enzyme and the regulatory sub-unit respectively. A third protein species at M_r 50 000, heavily phosphorylated but not cross-reacting (Fig. 2B) may be tentatively identified as the catalytic sub-unit.

The protein kinase preparation isolated from zucchini hypocotyls [14] does not undergo autophosphorylation but in some other aspects (e.g. phospholipid activation specificity) the enzyme appears to be similar to that studied here. Perhaps the major distinguishing characteristic of the *Amaranthus* enzyme, is the lowering of $[Ca^{2+}]$ K_m by diolein. This taken together with the cross reactivity to antiserum raised against bovine brain protein kinase C [15] and autophosphorylation of two of the cross-reacting species makes a compelling case for identification of this plant enzyme as the key regulatory enzyme protein kinase C. A biological role for calcium in hormone-dependent pigment formation in *A. tricolor* has already been demonstrated [21] and diacylglycerol-calcium synergism shown in the same process *in vivo* [2]. The properties of the protein kinase C described here suggests that the dual calcium-diacylglycerol activated pathway of stimulus–response coupling [4] also occurs in plants.

EXPERIMENTAL

Materials. Diolein, histone (type III-S), phosphatidylserine and leupeptin were from Sigma Chemical Co., St. Louis, U.S.A. Phosphatidylserine and diolein were stored as stock solns in

$CHCl_3$–MEOH (2:1) in the dark at −15°. Before use an aliquot was dried down under N_2 and sonicated in buffer to give a clear soln. Dephosphorylated casein was prepared as described [22]. [γ-^{32}P]-ATP (0.4–1 × 10^6 cpm/pmol) was prepared as described [23].

Enzyme purification. Amaranthus tricolor L. seeds were germinated in the dark at 25° for 88 hr as described [24]. Half seedlings (cotyledons plus the top 5 mm hypocotyl) were homogenised in 3 vol extraction medium at 2° using a Polytron homogenizer (3 × 15 sec) followed by several plunges of a tight fitting glass–teflon homogenizer. The medium [25] contained 0.25 M sucrose, 20 mM Hepes, pH 7.5, 2 mM EDTA, 5 mM EGTA 14 mM 2-mercaptoethanol and 0.01% leupeptin plus 2 mM PMSF. The homogenate was filtered through a sieve and the filtrate adjusted to pH 7.5 with NaOH. This was centrifuged at 100000 *g* for 60 min and the pellet discarded. The supernatant was filtered through Miracloth after removing the milky overlay. The filtrate was loaded onto a DEAE-Sephacel (Pharmacia) column (7 × 15 cm) pre-equilibrated with 10 mM HEPES buffer, pH 7.5, containing 2 mM EDTA, 5 mM EDTA and 10 mM 2-mercaptoethanol. Fractions (2 ml) were collected with a linear 0–0.8 M NaCl gradient at a rate of 40 ml/hr. All fraction tubes contained an aliquot of leupeptin (final concentration 10 μg/ml). Fractions containing Ca^{2+}/PS-dependent protein kinase were pooled, NaCl added to give a final concentration of 1.5 M, and loaded onto a Phenyl-Sepharose CL-4B (Pharmacia) column (7 × 1 cm) pre-equilibrated with 10 mM, pH 7.5, HEPES buffer containing 0.5 mM EDTA, 0.5 mM EGTA, 1.5 M NaCl and 10 mM 2-mecaptoethanol. The column was washed with a further 50 ml of the same buffer. Fractions were collected (2 ml, containing leupeptin, 10 μg/ml) with (1) a linear 1.5–0 M NaCl buffer gradient (40 ml), followed by (2) 40 ml M NaCl buffer. Flow rate was 40 ml/hr. Fractions with Ca^{2+}/PS-dependent protein kinase activity were pooled, concd using an Amicon ultrafiltration unit (PM 10 membrane) from 87 to 2 ml and chromatographed on Sephacryl S-200 (Pharmacia) equilibrated with 10 mM Hepes buffer, pH 7.5, 2 mM EDTA, 5 mM EGTA and 10 mM 2-mercaptoethanol. Bed vol. of this column was 120 ml and the flow rate 20 ml/hr. In some instances an affinity chromatography step was included. Material for this column was prepared by immobilizing phosphatidylserine and cholesterol in polyacrylamide [18]. On other occasions the Ca^{2+}-dependent binding of the enzyme to inside-out erythrocyte membrane vesicles was employed as a preliminary step [19].

Protein kinase assay. Assays contained 30 μl enzyme extract (either crude extract or column eluate), HEPES (30 mM, pH 7.5), [γ-^{32}-P]ATP (5 μM; 1000 cpm/pmol), $MgCl_2$ (5 mM), type III-S histone (167 μg/ml), EGTA and $CaCl_2$ in concentrations as given in legends, phosphatidylserine (40 μg/ml) and diolein (8 μg/ml) in a final volume of 120 μl. Free [Ca^{2+}] was calculated as described in the Addendum to ref. [13]. Incubation was at 30° for 10 min. Reactions were terminated and assayed by the P81 (Whatman) paper method [13].

Gel electrophoresis. Standard protein kinase assays (except for increased [γ-^{32}P] ATP sp. act. ~ 2000 cpm/pmol) were performed and the reaction stopped by adding 72 μl 2x Laemmli [26] sample buffer, 8 μl 2-mercaptoethanol and 5 μl 0.5% bromphenol blue. After boiling for 2 min, 60 μl sample was loaded per gel tract. For autophosphorylation experiments histone was omitted. Proteins were examined by SDS-PAGE using 12% resolving gel and 3% stacking gel [26]. For autoradiography gels were dried and exposed to Kodak Royal X-Omat film in the presence of a fluorescent intensifying screen.

REFERENCES

1. Hepler, P. K. and Wayne, R. O. (1985) *Annu. Rev. Plant Physiol.* **36**, 397.
2. Elliott, D. C. (1986) in *Molecular and Cellular Aspects of Calcium in Plant Development* (Trewavas, A., ed.) p. 285. Plenum Press, New York.
3. Dieter, P. (1984) *Plant, Cell Envir.* **7**, 371.
4. Nishizuka, Y. (1984) *Nature* **308**, 693.
5. Boss, W. F. and Massel, M. O. (1985) *Biochem. Biophys. Res. Commun.* **132**, 1018.
6. Sandelius, A. S. and Sommarin, M. (1986) *Plant Physiol.* **80S**, 80.
7. Helsper, J. P., de Groot, P. F., Linskens, H. F. and Jackson, J. F. (1986) *Phytochemistry* **25**, 2193.
8. Heim, S. and Wagner, K. G. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1175.
9. Irvine, R. F., Letcher, A. J. and Dawson, R. M. C. (1980) *Biochem. J.* **192**, 279.
10. Helsper, J. P., de Groot, P. F., Linskens, H. F., and Jackson, J. F. (1986) *Phytochemistry* **25**, 2053.
11. Morré, D. J., Gripshover, B., Monroe, A. and Morré, J. T. (1984) *J. Biol. Chem.* **259**, 15364.
12. Morré, D. J., Morré, J. T. and Yarnold, R. L. (1984) *Plant Physiol.* **75**, 265.
13. Elliott, D. C. and Skinner, J. D. (1986) *Phytochemistry* **25**, 39.
14. Schäfer, A., Bygrave, F., Matzenauer, S. and Marmé, D. (1985) *FEBS Letters* **187**, 25.
15. Elliott, D. C. and Kokke, Y. S. (1987) *Biochem. Biophys. Res. Commun.* (in press).
16. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986) *Science* **233**, 853.
17. Elliott, D. C. and Kokke, Y. S. (1986) *Contemporary Themes in Biochemistry* (Kon, O. L. *et al.*, eds) p. 370 Cambridge University Press, Cambridge.
18. Uchida, T. and Filburn, C. R. (1984) *J. Biol. Chem.* **259**, 12311.
19. Wolf, M., Cuatrecasas, P. and Sahyoun, N. (1985) *J. Biol. Chem.* **260**, 15718.
20. Ashendel, C. L. (1985) *Biochim. Biophys. Acta* **822**, 219.
21. Elliott, D. C. (1983) *Plant Physiol.* **72**, 215
22. Reimann, E. M., Walsh, D. A. and Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986.
23. Johnson, R. A. and Walseth, T. F. (1979) *Adv. Cyclic Nuc. Res.* **10**, 135.
24. Elliott, D. C. (1979) *Plant Physiol.* **63**, 269.
25. Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. and Nishizuka, Y. (1980) *Biochem. Biophys. Res. Commun.* **97**, 309.
26. Laemmli, U. K. (1970) *Nature* **227**, 680.