Chemistry of Natural Compounds, Vol. 30, No. 1, 1994

A Ca^{2+}-PHOSPHOLIPID-DEPENDENT PROTEIN KINASE FROM COTTONPLANT SHOOTS

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UDC 577.152.2

The Ca^{2+}-phospholipid-dependent protein kinase from cottonplant shoots was purified by chromatography on DEAE-Sepharose CL-6B, and then on phenyl-Sepharose CL-4B. According to electrophoresis in PAAG, the enzyme was practically homogeneous and had a molecular mass of ~57 kDa. In the presence of Ca^{2+} alone, the enzyme was activated to only a slight degree. Under the combined action of Ca^{2+} and a phospholipid the action of the enzyme rose severalfold. A determination of amino acid specificity showed that the protein kinase isolated was a serine- and threonine-specific protein kinase.

In recent years, great attention has been devoted to investigations of the realization of hormonal action. It is obvious that in plant cells, just as in animals cells, a leading role in the proceses of regulation is played by the reversible phosphorylation–dephosphorylation of proteins catalyzed by protein kinases and protein phosphatases [1]. The literature information that is accumulating is ever more strongly substantiating the key role of protein kinase C in these processes [2-6].

Protein kinase C is a unique enzyme among known kinases and exhibits its activity in the presence of both Ca^{2+} and a phospholipid [7-9]. Its presence in plant cells was shown in 1984 [10]. Although information on the transmission of signals in plants, particularly through Ca^{2+} switching [11] is continuously increasing, investigations in this field are only at the initial stage. In this connection, the identification of the components of the signal systems of plants taking part in the formation of the cell's response to an external action and their isolation and property characterization are a matter of urgency.

We have investigated the cytosolic, nuclear, and membrane fractions of a homogenate of three-day cottonplant shoots and have detected a Ca^{2+}-phospholipid-dependent protein kinase in all the fractions. In the presence of relatively high concentrations of Ca^{2+} (more than 10^{-4} M) the cytosolic protein kinase C was membrane-bound [9]; i.e, the ratio of the enzyme activities in the soluble and membrane fractions largely depends on the level of Ca^{2+} during homogenization.

In order to prevent the binding of the cytosolic protein kinase C with membranes and also to protect the enzyme from limited proteolysis by a Ca^{2+}-dependent neutral protease, in the purification of the enzyme it is necessary to use relatively high concentrations of metallocomplexones. Thus, for homogenization and the extraction of the enzyme we used a buffer containing 3 mM EGTA (buffer A). On homogenization in this buffer, the greatest activity was detected in the cytosolic fraction, and the protein kinase C was subsequently extracted from this.

The initial supernatant obtained after the centrifugation of the homogenate contained 20 mg of total protein. For the concentration and partial purification of the protein we carried out salt precipitation with ammonium sulfate, and, after dialysis, the whole of the precipitated protein was deposited on a column of DEAE-Sepharose CL-6B. The enzyme was eluted with a molarity gradient of NaCl (0–0.3 M). The increase in NaCl concentration within these limits led to the desorption of the active enzyme from the sorbent. The enzyme was eluted almost wholly at a NaCl molarity of 0.21-0.23 M (Fig. 1). This fraction, containing the active enzyme, was separated on a column of phenyl-Sepharose Cl-4B equilibrated with buffer B, with elution by a 0-70% gradient of isopropanol in the same buffer. The active fraction was then eluted at an isopropanol concentration of ~30% (Fig. 2). On electrophoresis in PAAG it showed a single protein band, which permitted the isolated enzyme to be considered homogeneous and enabled it to be characterized. From the electrophoretic results the molecular mass of the isolated enzyme was determined as ~57 kDa.

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 0009-3130/94/3001-0092$12.50 ©1994 Plenum Publishing Corporation

TABLE 1

Activator	Activity, pulses/min
– Ca^{2+} – phosphatidylcholine	450
+ Ca^{2+} – phosphatidylcholine	470
– Ca^{2+} – phosphatidylcholine	480
+ Ca^{2+} + phosphatidylcholine	2800

TABLE 2

Activator	Activity, pulses/min
Phosphatidylcholine	2800
Phosphatidylserine	2450
Phosphatidylinositol	1680
Cardiolipin	1430
Lysophosphatidylcholine	1020

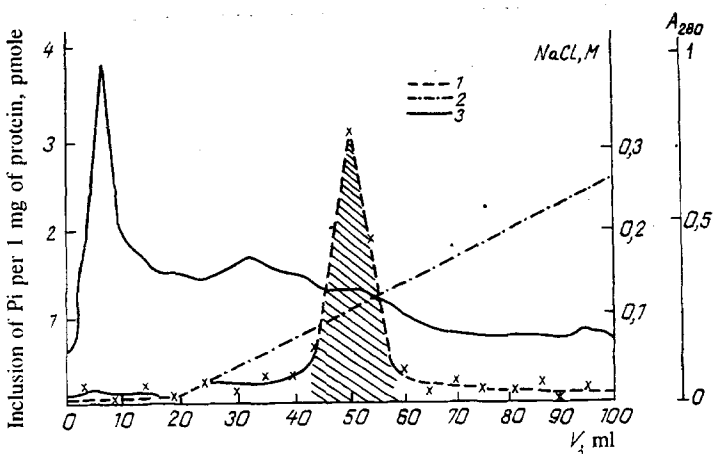

Fig. 1. Ion-exchange chromatography on DEAE-Sepharose CL-6B: 1) protein kinase activity; 2) NaCl concentration; 3) absorption at 280 nm.

The effect of various activators on protein kinase activity was investigated. In the presence of Ca^{2+} ions the enzyme was activated to only a slight degree (Table 1). Phospholipid (phosphatidylcholine) by itself did not activate the enzyme in the absence of Ca^{2+} ions. On the combined action of Ca^{2+} and phosphatidylcholine the enzyme was activated severalfold in comparison with the activity observed in the presence of Ca^{2+} ions alone. In an investigation of the influence of various phospholipids on the enzymatic activity it was found that the most pronounced stimulating action was possessed by phosphatidylcholine. The activating properties fell in the sequence phosphatidylserine, phosphatidylinositol, cardiolipin, lysophosphatidylcholine (Table 2).

It is known that a diacylglycerol, one of the products of the decomposition of the membrane phospholipids, raises the affinity of the enzymes with cofactors, thereby increasing the activity of the Ca^{2+}-phospholipid-dependent protein kinases [5, 6]. The influence of 1,2-diolein on the protein kinase activity was investigated, and it was shown that the activity of the enzyme increased severalfold on the addition of only a few micrograms of diolein to the reaction mixture (Table 3).

TABLE 3.

Activator	Activity, pulses/min
Ca^{2+} + phospholipid (phosphatidylcholine)	2800
Ca^{2+} + phosphatidylcholine + 1,2-diolein (2 μg)	9500

In all the experiments to determine the activity of the enzyme, incubation was carried out for 1 min at room temperature. On more prolonged incubation the activity of protein phosphatases appeared clearly (Fig. 3). Moreover, this activity increased considerably when calcium ions were present in the reaction mxiture.

The amino acid specificity of the protein kinase was determined as described in the Experimental part, the fractionation of the products of the partial acid hydrolysis of the phosphorylated substrate being achieved by high-voltage electrophoresis. From the results of electrophoresis we concluded that the Ca^{2+}-phospholipid-dependent protein kinase that we had isolated was a serine-threonine-specific protein kinase.

Although the facts presented give clear proofs of the presence in the cotton plant of a Ca^{2+}-phospholipid-dependent protein kinase and a Ca^{2+}-dependent protein phosphatase, the physiological role of these enzymes has not been determined.

EXPERIMENTAL

We used phosphatidylserine and 1,2-dioleoyl-sn-glycerol from Sigma (USA), [γ-^{32}P]ATP from the Radiopreparat production combine (Tashkent), phenylmethanesulfonyl fluoride, Tris, EGTA, 2-mercaptoethanol, phenyl-Sepharose, and DEAE-Sepharose from Pharmacia (Sweden), phosphocellulose P-11 from Whatman (United Kingdom), phosphoamino acids from Serva (FRG), electrophoretic reagents from Sigma (USA), and a set of standard proteins for molecular mass determination from Pharmacia (Sweden); all the other reagents were of domestic production and of os. ch. ["ultrapure"] grade. All solutions were prepared in deionized water.

Isolation of the Protein Kinase C. Seeds of a C-6254 cotton plant that had previously been treated with concentrated sulfuric acid to eliminate fibers and washed with water to neutrality were steeped in water at room temperature for a day and were then germinated in rolls of moistened filter paper in the dark at 27-28°C for two days. The shoots were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 3 mM EGTA, 2 mM PMSF, 50 mM 2-mercaptoethanol, and 0.26 M sucrose (buffer A) in a Potter homogenizer at a ratio of 2 ml of buffer per 1 g of crude weight of the shoots.

Homogenization and all the subsequent stages of the purification of the protein kinase C were carried out at 4°C. Extraction was performed for one hour with constant stirring by a magnetic stirrer. After the end of extraction the homogenate was filtered through two layers of Kapron [polycaprolactam] and was cenrifuged at 18,000 rpm for 30 min. The deposit was discarded, and the supernatant was used for the subsequent isolation of the enzyme.

To concentrate the protein in the supernatant we used salt precipitation with $(NH_4)_2SO_4$ to 100% concentration. The precipitate was allowed to form overnight. Then it was separated from the supernatant, dissolved in Tris-HCl buffer (pH 7.5), and dialyzed against water for a day with three changes of the dialysis liquid, and in a fourth stage it was dialyzed against 50 mM Tris-HCl, pH 7.5.

The ion-exchange chromatography of the protein fractions precipitated by ammonium sulfate was conducted on a column (1. 5 × 8 cm) of DEAE-Sepharose CL-6B using a molarity gradient of NaCl of from 0 to 0.3 M in 0.05 M Tris-HCl buffer, pH 7.5. The rate of flow was 30 ml/h, and the fraction volume 1 ml. Each fifth fraction was tested for protein kinase activity.

The hydrophobic chromatography of the active fraction obtained after the separation of the proteins on DEAE-Sepharose was conducted on a 1 × 6 cm column of phenyl-Sepharose CL-4B equilibrated with 0. 3 M NaCl in 50 mM Tris-HCl, pH 7.5 (buffer A)). A molarity gradient of NaCl of from 0.3 to 0 M and then a gradient of isopropanol of from 0 to 70% in the same buffer were used.

Protein kinase C activity was determined by the method of [28] [sic]. A medium with the following composition was used: 50 mM Tris-HCl, pH 7.5; 0.5 mM $MgCl_2$; 40 μg of substrate; 20 μg of phospholipid; and 50 μmole of [γ-^{32}P]ATP. The volume of the reaction mixture was 200 μl. The reaction was started by the addition of the enzyme solution (50 μl). After the end of incubation, a 10-μl aliquot from the reaction mxture was deposited on phosphocellulose P-11 and was washed with

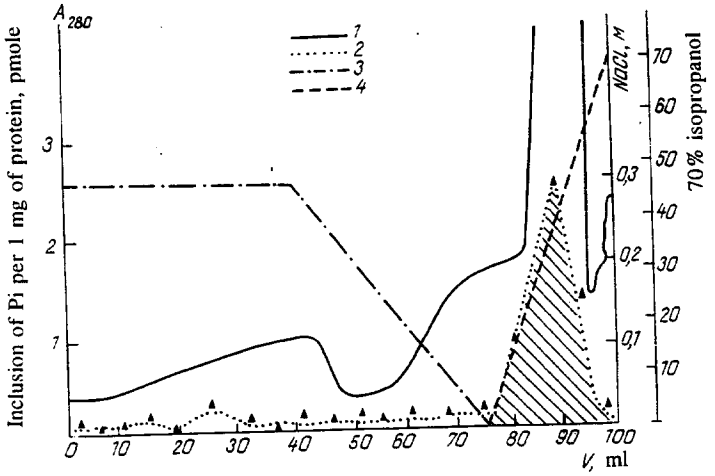

Fig. 2. Hydrophobic chromatography on phenyl-Sepharose CL-4B: 1) absorption at 280 nm; 2) protein kinase activity; 3) NaCl concentration; 4) isopropanol concentration.

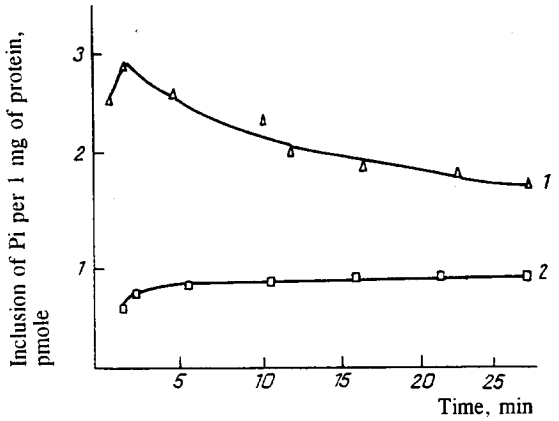

Fig. 3. Protein kinase activity of the cytosolic fraction of a homogenate of cottonplant shoots in the presence (1) and in the absence (2) of calcium chloride (0.5 mM).

four volumes of 5% TCA and then with four volums of ethanol, and the filters were dried and counted in a scintillation counter.

Electrophoresis was conducted by Laemmli's method [12] on 9 × 12 × 0. 6 [cm (?)] plates in the presence of 0.1% of Na-SDS.

Protein was determined quantitatively by Lowry's method [13], with the use of BSA as standard.

Th molecular mass of the enzyme was determined by comparing its electrophoretic mobility with those of marker proteins in a parallel PAAG lane.

The amino acid specificity was determined by a modified method [14]. The PAAG band corresponding to the phosphorylated protein was homogenized in 0.02 M Tris-HCl buffer (pH 7.5), and then the supernatant obtained after centrifugation was treated with 5% of TCA and was centrifuged again. The deposit was washed with 500 μl of a 2:1 mixture of chloroform and methanol and, after drying, was dissolved in 200 μl of 5.7 N HCl. The solution was thermostated in sealed tubes at 110°C for 1 h, and then the samples were dried and were deposited on cellulose plates with markers: phosphoserine, phosphothreonine, and phosphotyrosine in an amount of 10-15 μg per point, and high-voltage electrophoresis was conducted in the pyridine–acetic acid–water (10:100:1890), pH 3.5, system at 900 V for 1 h. After electrophoresis, the plate was dried and subjected to autoradiography at −20°C overnight on a RT-1 x-ray film, after which the spots were revealed with 0.2% ninhydrin.

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