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SOME CHARACTERISTICS OF COTTON PLANT PROTEIN KINASE C

V. V. Kim[a], K. S. Takhtobin[b], Zh. Abdurakhmanova[a], and Sh. I. Salikov[a]

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The change in the activity of the protein kinase C of cotton plant shoots as functions of the temperature, the time of incubation, and the concentration of Mg^{2+} ions has been investigated. It has been established that the maximum activity is shown at a temperature of 25-40°C, an incubation time of 30 min, pH 7.0, and a Mg^{2+} concentration of 10 mM.

We have previously described a method for isolating protein kinase C from three-day cotton plant shoots [1]. In the present paper we give the results of a determination of the optimum conditions for the Ca^{+}-phospholipid-dependent phosphorylation of the proteins of a homogenate of three-day cottonplant shoots.

In preceding experiments, the concentration of $MgCl_2$ in the determination of the activity of protein kinase C was 0.5 mM, with 2 mM $CaCl_2$, 50 mM (γ^{32}p)-ATP, 20 μg of phospholipid, and 40 μg of histone H1. The activity of protein kinase C depends greatly on the concentration of magnesium ions (Fig. 1). Thus, with a doubling of the concentration of magnesium ions the activity of protein kinase C scarcely changed and it increased only slightly at a concentration of 10 mM. At a magnesium ion concentration of 50 mM, the activity of protein kinase C reached a maximum, and at 100 mM we observed a fall in activity to 67% of the maximum.

Electrophoresis in the Laemmli system [2] showed qualitative differences in the compositions of the phosphorylated proteins as a function of the concentration of magnesium ions (1, 10, 20, and 50 mM). In almost all the lanes, phosphorylated proteins with molecular masses of 80, 30, and 23 kDa were observed. From a concentration of 10 mM, a protein with a molecular mass of 33 kDa was phosphorylated with particular intensity. The variability of the phosphorylation of a protein with a molecular mass of 40 kDa is interesting: this was phosphorylated to different degrees at 10 and 20 mM $MgCl_2$ while it almost disappeared at a concentration of 50 mM. Particular interest is presented by a protein with a molecular mass of 107 kDa which was phosphorylated only at a magnesium ion concentration of 20 mM. The qualitative differences in the phosphorylation of the proteins of cottonplant shoots in the presence of different concentrations of magnesium ions show its capacity for affecting the activity of protein kinases either directly or indirectly and their substrate specificity.

The value of K_M for histone H1 calculated from the Michaelis-Menten equation was 42 μg/ml, while for animal tissues this constant is 50 μg/ml [3]. The optimum pH of protein kinase C is 7.0 (Fig. 3). The temperature optimum is from 25 to 40°C, the activity of the enzyme falling sharply at temperatures above 40°C (Fig. 4). The enzyme is inactivated completely at a temperature of about 75°C.

It is known that enzymes possess a high degree of conservatism in their structures, which is possibly responsible for the common features of the mechanism of their action regardless of the sources from which the enzymes were isolated. Thus, we have shown for the first time with the aid of electrophoresis in polyacrylamide gel and by immunoblotting that the cotton plant protein kinase C that we had isolated and the protein kinase C from cattle brain contain in their structures determinants with similar antigenic properties (Fig. 5). In this experiment, we used monoclonal antibodies of clone MC 5, code RP N 536 presented for S. E. Severin by Syl'via Kasoblan*. An immunoblot of protein kinase C and a homogenate of cottonplant shoots showed that the monoclonal antibodies detected a polypeptide with a molecular mass of 57 kDa in both cases.

*Direct transliteration of unidentified name — Translator.

a) A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 62 70 71. b) Institute of Nuclear Physics, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 44 26 03. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 141-145, January-February, 1995. Original article submitted October 17, 1994.

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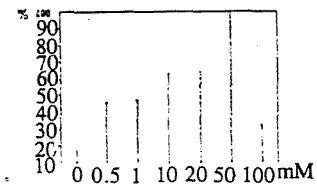

Fig. 1

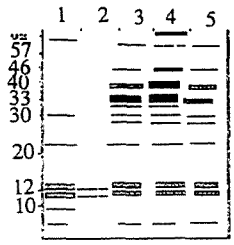

Fig. 2

Fig. 1. Dependence of protein kinase C activity on the concentration of Mg ions.

Fig. 2. Electrophoretogram of the phosphorylated proteins of cotton plant shoots in dependence on the concentration of magnesium ions.

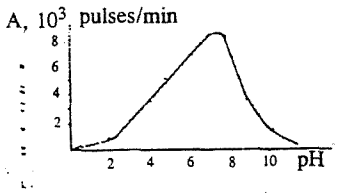

Fig. 3

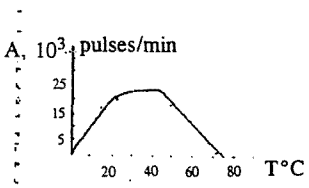

Fig. 4

Fig. 3. Dependence of the activity of protein kinase C on the pH of the incubation medium.

Fig. 4. Dependence of the activity of protein kinase C on the temperature.

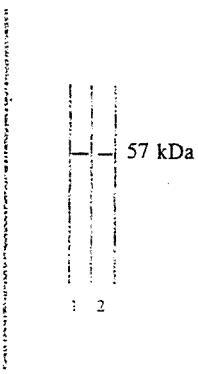

Fig. 5

Fig. 6

Fig. 5. Immunoblots of PKC (*1*) and of a homogenate of three-day cotton plant shoots (*2*).

Fig. 6. Electrophoretic analysis of the phosphorylation of the proteins of a homogenate of three-day cotton plant shoots without PKC cofactors (*1*) and in the presence of Ca^{2+} and phosphatidylserine (*2*).

Protein kinase C is very sensitive to proteolysis, particularly by a Ca^{2+}-dependent neutral thiol protease [4, 5], and therefore in all experiments, just as in the isolation of the enzyme, we used leupeptin — an inhibitor of this protease.

The facts given characterize the properties of protein kinase C from the material that we investigated. However, in addition to the protein kinase C the presence of which we judged from the stimulation of activity, the homogenate contained other protein kinases the activity of which was also affected by calcium ions. It was therefore of great interest to study the influence of Ca^{2+} and phospholipid on the phosphorylation of individual polypeptides (Fig. 6).

In the first lane (Fig. 6) are shown the bands of the phosphorylation of proteins without cofactors when the phosphorylation of a homogenate of three-day shoots was carried out in the absence of Ca^{2+} ions and of phospholipid. The

second lane shows the phosphorylation of polypeptides in the presence of Ca^{2+} and phosphotidylserine. The results indicate that Ca^{2+} and phospholipid can activate the dephosphorylation of polypeptides with molecular masses of 46, 41, 38, 30, and 23 kDa. No phosphorylation of proteins with molecular masses of 46, 41, and 38 kDa was observed without Ca^{2+} and phospholipid, i.e., it was induced by Ca^{2+} ions and phospholipid. In addition to this, the level of phosphorylation of the proteins with molecular masses of 30 and 23 kDa increased, in complete agreement with results obtained previously.

Cofactor-independent phosphorylation was determined under the conditions of [1] but without Ca^{2+} and phosphatidylserine.

EXPERIMENTAL

We used phosphatidylserine from Sigma (USA), [γ-^{32}P)-ATP from the Tashkent Radiopreparat Industrial Combine, phosphocellulose P-11 from Whatman (United Kingdom), electrophoretic reagents from Sigma (USA), and a set of standard proteins for molecular mass determination from Pharmacia (Sweden).

The activity of protein kinase C was determined as described previously [1].

Immunoblotting was carried out in the following sequence:

1. A homogenate of three-day cottonplant shoots was prepared by grinding the shoots in a mortar at 4°C in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM PMSF, 0.25 M sucrose, 5 mM 2-mercaptoethanol, and 3 mM EGTA. The homogenate was centrifuged at 10,000 *g* for 20 min, and the supernatant was used for further separation by electrophoresis.
2. The electrophoretic analysis of the proteins was conducted in an exponential gradient (7-15%) of a polyacrylamide gel at a constant current of 25 mA per plate (6:9:0.1 cm).
3. Transfer of the proteins from the PAAG to a nitrocellulose membrane.
4. Blocking of the nonspecific sites on the membrane with 3% BSA in PBS.
5. Incubation of the proteins on the membrane with MC5 monoclonal antibodies in 0.1% w/v BSA in PBS (1 h at 37°C).
6. Washing of the membrane with 0.1% Tween in PBS, 5 min, four times.
7. Incubation of the membrane with antitarget Ig-peroxidase conjugate diluted 1:100 with 0.1% BSA w/v in PBS, 30 min at room temperature.
8. Washing of the membrane with 0.1% Tween in PBS, 5 min, four times.
9. Incubation of the membrane with 0.5 mg/ml of 3,3′-diaminobenzidine in PBS. A solution of H_2O_2 was added immediately before use, bringing the concentration to 0.3% v/v.
10. The membrane with the colored protein band was washed with water and dried.

One-dimensional electrophoresis in PAAG for the separation of the phosphorylated proteins was conducted by Laemmli's method [2] in 10% gel.

Autoradiographic exposure was made on a RD-1 X-ray plate at 20°C for two days.

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