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PROTEIN KINASE FROM COTTON SEEDS

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A Ca^{2+}-dependent protein kinase C, active at pH 6.5-8.0 has been found in cotton seeds for the first time. The localization of the enzyme in the seeds has been established and some of its properties are described (stability in various media, capacity for performing the phosphorylation of various substrates, activation by calcium ions). Highly active preparations of cottonseed protein kinase C have been isolated by biospecific chromatography.

Phosphorylation-dephosphorylation reactions are the main link in the transformation of a hormonal signal into specific metabolic responses [1, 2].

The enzyme protein kinase C performs the phosphorylation of cell proteins, thus mediating the action of hormones on the effector systems of the cells. In animal tissues, this enzyme plays the main role as part of the transmembrane "signal systems" [3] and controls many cell functions [4-6].

In plant cells, as in animal cells, protein kinase C is a mediator and enhancer of the action of hormones [7-9]. In spite of the factual material that has accumulated indicating the importance of the phosphorylation of proteins for the control of metabolic processes in higher plants [10-12], investigations in this field are only in their initial stage.

We have established the presence in cotton seeds of an enzyme with protein kinase activity. The dependence of the enzymatic activity on the pH was investigated in the extract obtained after the centrifugation of a suspension of three-day cotton seedlings in a suitable buffer (1:5). The phosphorylation of casein took place with the maximum rate at pH 6.5-8.0. The enzymatic activities were concentrated almost completely in the soluble fraction.

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Fraction	Specific Activity 6.5	at pH 8.0
Homogenate	116	230
Mitochondrial fraction	0	0
Soluble fraction	220	324

The protein kinase active at pH 6.5-8.0 was best extracted into a Tris-HCl buffer, 0.05 M, pH 6.5, containing 0.1 M KCl, and into Tris-HCl buffer, 0.05 M, pH 8.0, containing 0.25 M NaCl and 0.25 M $(NH_4)_2SO_4$. In these buffers, the pH optima of the enzyme are 6.5 and 8.0, respectively:

Extraction conditions	Extracted protein, mg	Specific activity
Tris-HCl buffer 0,05 M, pH 7.5	150	110
" 0,05 M, pH 7,5, containing 0,1 M NaCl	200	140
" 0,05 M, pH 6,5, containing 0,1 M NaCl	200	190
" 0,05 M, pH 6,5, containing 0,1 M KCl	300	290
" 0,05 M, pH 6,5, containing 0.025 M sucrose and 0.01 M DTT.	750	210
" 0,05 M, pH 8,0, containing 0,25 M NaCl and 0,25 M $(NH_4)_2SO_4$	700	470

The protein kinase retained its activity on incubation in 0.05 M Tris-HCl buffer, pH 6.5, containing 0.1 M KCl at 0°C for 72 h. The stability of the protein kinase depended on the medium in which the enzyme was extracted. Thus, it increased appreciably in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.25 M NaCl and 0.25 M $(NH_4)_2SO_4$. In this buffer, the protein kinase was stable at 0° for four days, i.e., a rise in the ionic strength of the solution led to an increase in the stability of the enzyme.

At pH 6.5, the protein kinase catalyzed the phosphorylation not only of casein but also of other substrates (the concentrations of all the substrates were 100 μg; the mean values of three experiments are given:

Substrate	Specific Activity	In % on casein
Casein	350	100
Histone H1	180	51
BSA	220	62
Cottonseed globulin	326	93

On passing from bovine serum albumin (soluble substrate) to the globulin (insoluble) the activity of the protein kinase rose, which indicated a specificity of the cottonseed kinase towards its natural substrate – cottonseed globulin.

The rate of the phosphorylation of the substrate by the cottonseed protein kinase did not depend on the time of incubation with the substrate: $A_{15\ min}$ = 350 pmole of phosphorus/min/mg of protein, and $A_{60\ min}$ = 345 pmole of phosphorus/min/mg of protein.

The thermal stability of the protein kinase increased in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.25 M NaCl and 0.25 M $(NH_4)_2SO_4$, and we used this fact to develop a method for purifying the enzyme.

The known methods for purifying a protein kinase are laborious and multistage, and the yield of enzyme is very low. They frequently presuppose the synthesis of sorbents with particular ligand groups, which is also fairly complicated [10-12].

We have proposed a simple, rapid, and accessible method for the biospecific purification of the protein kinase on a sorbent – sorsilen – impregnated with proteins which is based on the capacity of protein kinases for exhibiting a high affinity for protein substrates of the type of casein or albumin.

TABLE 1. Influence of a Number of Conditions on the Binding of the Protein Kinase with a Biospecific Sorbent (Sorsilen impregnated with BSA) and the Conditions of Elution in the Desorption of the Enzyme

Conditions for the adsorption and the elution of the enzyme	Protein, mg	Activity		Yield as activity, %	Yield as protein, %
		total	spec.		
Adsorption					
Tris-HCl buffer 0,05 M, pH 8,0 contg. 0,25 M NaCl and 0,25 M $(NH_4)_2SO_4$	40	11040	276		
Elution					
Tris-HCl buffer 0,05 M, pH 8,0, contg. 0,5 M NaCl	3,05	6628	2173	51	7,6
Adsorption					
Tris-HCl buffer 0,05 M, pH 8,0	65	7150	110		
Elution					
Tris-HCl buffer 0,05 M, pH 8,0, contg. 0,5 M NaCl	2,24	1153	515	16	3,5
Adsorption					
Tris-HCl buffer 0,05 M, pH 7,5, contg. 2,5 mM $MgCl_2$, 50 mM KCl, 5 mM EDTA and 250 mM sucrose	51	1887	37		
Elution					
Tris-HCl buffer 0,05 M, pH 7,5, contg. 0,5 M NaCl	3,63	1234	340	65	7,2
Adsorption					
Tris-HCl buffer 0,05 M, pH 7,5 contg. 3 mM EDTA, 1 mM DTT and 250 mM sucrose	41	6886	146		
Elution					
Tris-HCl buffer 0,05 M, pH 7,5, contg. 0,5 M NaCl	2,42	2767	1146	40	5,9

In order to determine the optimum conditions for the biospecific chromatography of the protein kinase, we investigated the factors affecting the binding of the enzyme with the sorbent and the influence of the composition of the eluent on the desorption of the protein kinase from the biospecific sorbent. Washing the sorbent with the initial buffer led to the desorption of proteins that were apparently bound through nonspecific hydrophobic interactions. When the ionic strength of the eluting solvent was increased, the enzyme-ligand complex broke down. Desorption of the enzyme took place on elution with 0.1 M NaCl, but only 24% (in terms of activity) of the amount of enzyme absorbed was desorbed from the sorbent under these conditions. An increase in ionic strength favored the desorption of the protein kinase from the sorbent. A rise in the concentration of NaCl to 0.5 M led to the elution of the active protein (Table 1).

The procedure for purification on a biospecific sorbent that has been described ensures the isolation of a highly active enzyme preparation with a fairly high yield (from 5.9 to 7.6%) in one day. The preparations isolated are distinguished by affinity for immobilized albumin and a high maximum specific activity.

Since calcium ions are specific activators for the majority of protein kinases [3, 7], it appeared of interest to determine the action of calcium ions on the fractions possessing protein kinase activity that were isolated from the biospecific sorbent. The investigations showed an increase in protein kinase activity with an increase in the concentration of Ca^{2+} ions in the incubation medium, which permits this enzyme to be characterized as a protein kinase C:

Activating additives	without Ca^{2+}	50 μM Ca^{2+}	100 μM Ca^{2+}
Protein kinase activity, %	15	80	100

By selecting the concentration of $CaCl_2$ it was possible to activate the enzyme fairly effectively and, therefore, the activity of the protein kinase was subsequently measured in the presence of $CaCl_2$.

It was established that the protein fractions isolated from cotton seeds, while exhibiting affinity for a phytohormone, had a considerable protein kinase activity and that the latter depended on the calcium concentration. A further investigation of the phosphorylation of protein by cottonseed protein kinase C may be important both for characterizing the substrate specificity and for understanding the mechanism of the interaction of receptors for signal substances — hormones — with definite enzyme systems.

EXPERIMENTAL

The following reagents were used: dithiothreitol from Reanal, sorsilen from Czechoslovakia, and reagents of domestic manufacture of kh.ch. ["chemically pure"] or os.ch. ["ultrapure"] grade. All the solutions were prepared with double-deionized water. As the substrate proteins for the protein kinase C we used histone H1 isolated from calf thymus [13], casein and bovine serum albumin (BSA), and also cottonseed globulin obtained by the method of [14].

Protein was determined by Lowry's method [15], BSA being used as standard.

The protein kinase C activity was determined by a published method [11] in a medium with the following composition: 50 mM tris-HCl (pH 6.5-8.0) 10 mM $MgCl_2$, 50 μM ATP containing [γ^{32}P]ATP ($2 \cdot 10^5$ to $2.5 \cdot 10^5$ pulses/min in a 200-μl sample). The concentration of the substrate proteins was 100 μg. The reaction was began by the addition of the enzyme solution (from 0.05 to 0.1 ml). After the end of incubation (15 min at 28°C), the reaction mixture, in an amount of 20 μl, was deposited on filters (Whatman 3MM), and after several washings with 10% trichloroacetic acid solution and with 95% ethanol, the filters were dried and placed in a liquid scintillation counter. Specific activities were expressed in pmole of phosphorus transferred to 1 mg of protein in 1 min.

Preparation of the Biospecific Sorbent. A suspension of 1 g of sorsilen in 5 ml of a 1% solution of BSA in 0.05 M universal buffer, pH 3.5 was stirred and was kept at +4°C for 10-12 h. Then the sorbent was washed with the same buffer and was resuspended in the buffer in which the adsorption of the enzyme was to be carried out.

The enzyme was isolated with the aid of a combination of the procedures described in [7, 8, 11]. Three-day cotton seedlings of the variety 108-F (germinated in the dark at 28°C) (10 g) were homogenized in 50 ml of the appropriate buffer. Then extraction was carried out with stirring at 4°C for 40-60 min. The homogenate was filtered through Kapron [polycaproamide] fabric and was centrifuged at 25,000g for 1 h. The deposit was sucked off, and the supernatant liquid was used for the subsequent purification of the protein kinase.

Purification by Biospecific Chromatography. The crude protein kinase preparation (10 ml) was added to 1 g of sorsilen-BSA in 0.05 M Tris-HCl buffer (pH 7.5 or 8.0) with the appropriate additives. The suspension was stirred at 4°C for 15 min. Then the sorbent was washed with a fivefold amount of 0.05 M Tris-HCl buffer twice and the washed protein fraction was separated by centrifugation (8000g, 10 min). The washed sorbent was resuspended in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and the protein fraction was eluted, after which the bound protein kinase was desorbed on the sorbent with 0.05 M Tris-HCl buffer containing 0.5 M NaCl.

SUMMARY

A Ca^{2+}-dependent protein kinase C active at pH 6.5-8.0 has been detected in cotton seeds for the first time. The localization of the enzyme in the seed has been established and some of its properties have been described (stability in various media, capacity for performing the phosphorylation of various substrates, activation by calcium ions). Highly active preparations of cottonseed protein kinase C have been isolated by biospecific chromatography.

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MODIFICATION OF DNA BY PHOTOACTIVABLE ARYL AZIDES

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Features of the interaction with DNA of photoactivable aryl azides having various substituents in the aromatic ring have been studied. The nature of the interaction with DNA and the degree of its modification depend substantially on the nature of these substituents. The results obtained can be used for obtaining DNA probes bearing various marker groups.

The study and use of natural biological compounds is connected to a considerable degree with their capacity for being modified under the action of various chemical agents. In this respect, the interaction of photoactivable reagents such as aryl azides with nucleic acids has been studied comparatively little. These compounds, on irradiation with light of various wavelengths, generate highly reactive nitrenes [1, 2], which interact with nucleic acids [3, 4]. In the present paper, we report features of the interaction with DNA of photoactivable aryl azides having various substituents in the aromatic ring. It has been shown that the nature of the interaction with DNA and the degree of its modification by the aryl azides depend substantially on the nature of these substituents. The results obtained can be used to obtain DNA probes bearing various marker groups.

As photoactivable reagents we synthesized the compounds the structural formulas of which are shown in Fig. 1. The choice of compound was determined by the aims of the present investigation: to study the influence of a) the presence of a nitro group in the aromatic ring, and b) the nature of the radical R' on photolysis and the interaction of the products of the photolysis of the azides with single- and double-stranded forms of DNA on irradiation with visible light.

In the first stage of the work we studied the influence of substituents in the aryl azide and the composition of the buffer on the characteristics of photolysis. Below, we give the half-decomposition times of the azide (I) determined from the curve of the dependence of the decrease in the absorption of a solution at 268 nm on the time of irradiation using a number of light-filters:

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