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DETECTION AND SEPARATION OF TWO FORMS OF COTTON-PLANT PYROPHOSPHATASE

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Two pyrophosphatase isoenzymes with identical molecular weights but differing in the pH optima of their action and also in their senstivity to some bivalent metal ions have been isolated from extracts of the cotyledons of three-day cotton-plant shoots by gel filtration and ion-exchange chromatography.

Inorganic pyrophosphatase (EC 3.6.1.1.) is a polyfunctional enzyme. Its participation in the phosphorus metabolism and also in the energy metabolism of the cell is well known [1]. Recently, another function of the enzyme has been revealed which is connected with the utilization of condensed phosphates representing an effective form of phosphorus fertilizers for plants and, in particular, for the cotton plant [2]. The utilization of condensed phosphate takes place with the participation of enzymes liberated into the external medium by the roots of the plant and hydrolyzing pyrophosphatase bonds [3]. We have previously reported on the presence and isolation of a pyrophosphatase enzyme from cotton-plant tissues [4]. In the present paper we give information obtained in the purification of the enzyme. The object of the investigations were three-day shoots of cotton plants of variety 108-F.

Gel filtration of the enzyme extract gave two fractions possessing pyrophosphatase activity (Fig. 1). They were combined and concentrated and used for the subsequent chromatographic stages. It must be mentioned here that although the pyrophosphatase activity in fraction 1 was 1.5 times greater than in fraction 2, the specific acitivity of the former was far smaller than that of the latter. The use of this procedure permits a large amount of ballast substances to be eliminated, since in fraction 1, together with pyrophosphate activity, a very large amount of ATPase activity, exceeding the pyrophosphatase activity by a factor of 3-4, and also tripolyphosphatase and phosphatase activities were detected. Consequently, we subsequently investigated mainly the second fraction. When it was chromatographed on DEAE-cellulose, the proteins were separated in two peaks (Fig. 2). Pyrophosphatase activity was detected in fractions 6-10 and 17-18. At the same time, the specific activity of the enzyme increased several fold. Together with this, in fractions 6-10, with a fairly high specific pyrophosphatase activity, ATPase activity (10-14% of the pyrophosphatase activity) was also detected. Conversely, fractions 8-10 possessed a high pyrophosphatase activity unaccompanied by other activities. When they were rechromatographed (Fig. 3), a single protein peak possessing only pyrophosphatase activity and eluting at a low ionic strength of NaCl was obtained. The enzyme exhibited its maximum activity at pH 8.6, and the addition of activator metals ($MgCl_2$) to it led to a six- to seven-fold increase in the specific activity of the enzyme. The enzyme eluted in fractions 17-18, in contrast to that in fractions 17–18, was active in the acid pH range and did not require the addition of activator metals for the manifestation of its activity. Furthermore, this pyrophosphatase was capable of hydrolyzing p-nitrophenyl phosphate well:

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Substrate hydrolyzed	Alkaline pyrophosphate, %	Acid pyrophosphatase, %
Pyrophosphate	100	100
Tripolyphosphate	1.8	10.5
Polyphosphate p-60	0.8	5.1
Polyphosphate p-110	0.6	5.1
Polyphosphate p-290	0.5	4.9
p-Nitrophenyl phosphate	3.6	19.6
Adenosine triphosphate	3.2	10.6
Adenosine diphosphate	8.1	30.1
Cytosine triphosphate	9.2	23.2
Guanidine triphosphate	8.3	14.8
Uridine triphosphate	9.8	27.0

Electrophoretic analysis of the first fraction (alkaline pyrophosphatase) revealed the presence of three protein fractions, and that of the acid fraction showed two (Fig. 4). It must be mentioned that the Rf values of the protein bands in the gel did not agree for the two fractions, which is an additional proof of the separation of the alkaline and acid pyrophosphatases from one another in the fractionation process.

EXPERIMENTAL

The enzyme extract was obtained by grinding the frozen shoots with glass powder in a small volume of Tris-HCl buffer, pH 7.2, followed by centrifugation at 10,000g with cooling (0-2°C) for 30 min. The precipitate was discarded, and the supernatant liquid was used for the subsequent investigations. The liquid obtained was treated with two volumes of cooled acetone and was left for 2 h for the complete precipitation of proteins. The precipitate formed was collected by centrifugation in the cold at 6000g. The resulting precipitate was dissolved in a small volume of the initial buffer and was centrifuged at 10,000g. The supernatant was dialyzed against distilled water at 2-4°C for 16-18 h. The precipitate formed on dialysis was also removed by centrifugation.

Pyrophosphatase activity was determined from the amount of orthophosphatase formed in an incubation mixture. The incubation mixture contained 0.2 ml of 0.05 M Tris-HCl buffer, pH 7.2, 0.1 ml of 0.005 M magnesium chloride, 0.1 ml of Sigma sodium pyrophosphate solution (1.5 mg/ml), and 0.1 ml of the enzyme preparation. The mixture was incubated at 37°C for 20 min, and the reaction was stopped by the addition of 0.5 ml of 0.5 M $HClO_4$. In the control test-tubes, the reaction mixture was first incubated and the protein solution was added after the reaction had been stopped. The specific activity of the enzyme was expressed in mU (amount of enzyme catalyzing the formation of 1 μm of orthophosphate liberated after 1 min under the given conditions, referred to 1 mg of protein in the enzyme preparation). Orthophosphate was determined by the method of Berenblum and Chain in the modification due to Weil-Malherbe and Green [5]. Protein was determined by Lowry's method [6].

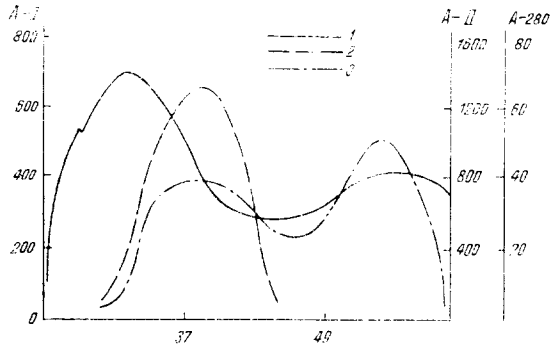
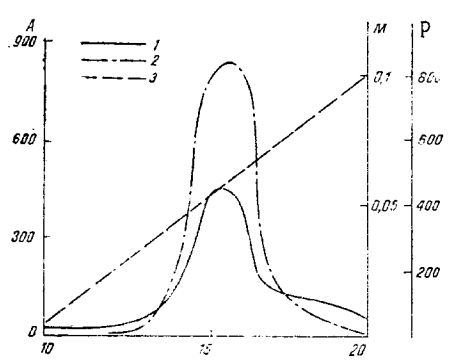


Fig. 1. Gel filtration of an enzyme extract from cotton-plant shoots on a column of Sephadex G-100: 1) elution profile at 280 nm; 2) ATPase activity; 3) pyrophosphatase activity; A-1) specific pyrophosphatase activity; A-2) specific ATPase activity (activities in μmole of P_i/mg in 1 min).



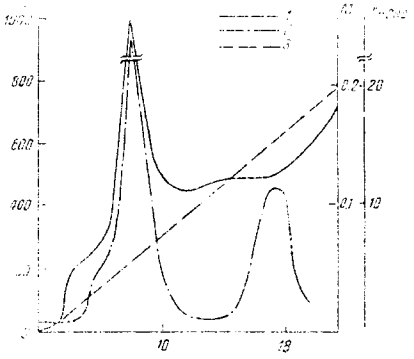


Fig. 2 Fig. 3

Fig. 2. Chromatography of the pyrophosphatases on DEAE-cellulose: 1) elution profile at 280 nm; 2) pyrophosphatase activity; 3) NaCl gradient.

Fig. 3. Rechromatography of the pyrophosphatase on a column of DEAE-cellulose DE-52: 1) protein content; 2) pyrophosphatase activity; 3) NaCl gradient.

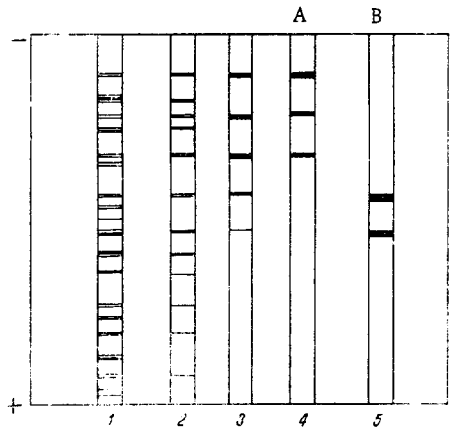


Fig. 4. Separation of the proteins in polyacrylamide gel (scheme). 1) Cell-free extract; 2) after gel filtration; 3, 5) after chromatography; 4) after rechromatography; A) alkaline pyrophosphatase; B) acid pyrophosphatase.

The concentration of the protein extract was carried out in cells with a Amicon membranes, which pass proteins with molecular weights up to 1 kD, under a pressure of gaseous nitrogen of 3-4 atm. The gel filtration of the enzyme extract was carried out with the use of Sephadex G-100 (Pharmacia) on a 1.9 × 70 cm column equilibrated with Tris-HCl buffer, pH 7.2. The rate of elution (6.6 ml/h) was regulated with the aid of a Uvicord III automatic pump. The protein extract was chromatographed on a column filled with DEAE-cellulose (Serva) equilibrated with Tris-HCl buffer, pH 7.2 (Cl^- form). Elution was carried out with a linear concentration gradient of NaCl at a rate of 6 ml/h. Rechromatography was performed with the aid of Whatman DEAE-cellulose DE-52 (first fraction). The protein fractions were recorded with the aid of the Uvicord-III instrument. Analytical electrophoresis of the enzyme preparations was performed in 7.5% polyacrylamide gel at pH 8.9 [7] with the aid of Reanal reagents. Each sample contained 100-150 µg of protein, and the current strength was 4 mA per tube.

SUMMARY

Two pyrophosphatase isoenzymes with identical molecular weights but differing in the pH optima of their action and also in their sensitivities to certain bivalent metal ions have been isolated form the cotyledons of three-day cotton-plant shoots.

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PREPARATION OF HOMOGENEOUS PROTAMINES BY THE FRACTIONATION OF NUCLEOPROTAMINE

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A new method has been developed for obtaining individual protamines which is based on the chromatographic separation of an ultrasonically treated nucleoprotamine solution. The separation of the nucleoprotamine from the gonads of the Caspian sturgeon Acipenser stellatus on CM-Sephadex G-25 led to the isolation of three fractions. Analysis showed that two fractions contained homogeneous protamine – stellins A and B. The third fraction contained nonprotamines and DNA.

Protamines form a peculiar group of basic nuclear proteins which, in the mature sex cells of a number of organisms (molluscs, amphibia, some reptiles, and also in the majority of fish) are bound into a strong complex with DNA. It is considered that the main function of the protamines is the compactization of the DNA and its protection from various physicochemical actions [1, 2]. Characteristic features of the protamines are their high content of basic amino acids, their limited set of neutral amino acids, and their low molecular weight ($\sim 5 \cdot 10^3$ daltons). As a rule, each species of fish is characterized by its own unique set of protamines consisting of from two to four proteins. The isolation of the individual proteins is one of the important problems arising in the study of the protamines.

The methods for obtaining homogeneous protamines used at the present time generally consist of two stages: 1) the isolation of the total proteins from the nuclear protamines; and 2) the fractionation of the mixture of proteins isolated with the aim of obtaining homogeneous protamines.

Extraction with mineral acids is used most frequently for the isolation of the total protamines [1, 3, 4]. This extremely simple method has a number of disadvantages. In the first place, the destruction of the protamines in the acid medium is possible. In the second place, other basic proteins, and also degradation products of nucleic acids are coextracted with the protamines. In the third place, repeated extraction is used for the complete isolation of the protamines. It is obvious that it would be extremely desirable to eliminate or improve this stage in the isolation of homogeneous protamines.

The second stage – the isolation of individual proteins – is usually performed with the aid of chromatography on cation-exchanging celluloses or Sephadexes [1, 4-6].

We have succeeded in simplifying the method of obtaining homogeneous protamines by excluding the stage of isolating the total proteins.

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