The experiments on binding were carried out by the method of filtration on Synpor nitrocellulose filters (Czechoslovakia). Microtest-tubes with a volume of 200 ml were each charged with 10 µl of ^{3}H-IAA or ^{3}H-BAP in a concentration of 10^{-6} N, 10 µl of water (in the control, 10 µmole of unlabeled phytohormone in a concentration of 10^{-3} M), and 80 µl of the solution of the protein under investigation. The mixture was incubated at 20°C for 30 min and was filtered under vacuum. The filters were washed with water and dried, and their radioactivities were determined in a ZhS-107 toluene scintillator on a Beta-1 counter.

Protein concentrations were determined by Bradford's method [6].

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ISOLATION AND CHARACTERIZATION OF PHYTOKININ-BINDING PROTEINS FROM THE COTTON PLANT

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UDC 547.857.581.192.7

A highly purified site of a cytokinin-binding protein with a K_D value for ^{3}H-BAP of 4.3 nM has been isolated from cotton-plant seedlings by the methods of biospecific and hydrophobic chromatography. According to the results of electrophoretic analysis under denaturing conditions its molecular mass is 43 kDa.

Cytokinin-binding proteins (CBPs) of plants play an important role in the molecular mechanism of the reception and transmission of a hormonal signal. It is assumed that they form with cytokinin a hormone-receptor complex which initiates a cascade of biochemical reactions determining the physiological response of the plant organism to the action of the given hormone.

The first communication on the isolation of proteins reversibly binding with kinetin and with benzylaminopurine (BAP) appeared in 1970 [1]. At the present time, voluminous experimental material has accumulated on the isolation and characterization of CBPs from various plants – wheat, barley, maize, mungo beans, squash, tobacco. It has been shown that it is mainly soluble CBPs with molecular masses of 40-45 kDa and binding constants with zeatin, K_D, of 10^{-7}-10^{-8} M, that are localized in the vegetative organs of cereals [2, 3]. In addition to soluble proteins, CBP sites have been detected in fractions of the endoplasmic reticulum [4].

The present work was devoted to the isolation, chromatographic purification, and characterization of the CBPs of the water-soluble fraction of proteins from cotton seedlings.

One the problems arising in the isolation of proteins from the cotton plant is the presence in a plant tissue homogenate of a large amount of various low-molecular-mass compounds, some of which – tannins, phenols, phytoalexins – may interact with proteins and inactivate them. Furthermore, the extract also contains endogenous cytokinins saturating the active sections of the receptor proteins and preventing their binding with the ligands of biospeci-

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TABLE 1. Isolation of Cytokinin-binding Proteins by Hydrophobic Chromatography on Polikhrom-1

Stage of isolation	Amount of protein, mg	Specific binding activity, nmole/mg
Homogenate	—	0,00008
Total water-soluble protein	40	0,006
20% ethanol eluate	0,188	—
" 40% "	0,180	0,058
" 60% "	0,210	0,062
" 80% "	0,282	0,043
" 96% "	0,353	0,008

TABLE 2. Isolation of the CBP by gel Chromatography on TSK-HW-65-F

Fraction	Amount of protein, mg	Specific binding activity, nmole/mg of protein
1	15,6	0
2	0,024	0,015
3	0,054	0,12
4	0,032	0,41
5	0,033	0,78
6	0,220	0,06

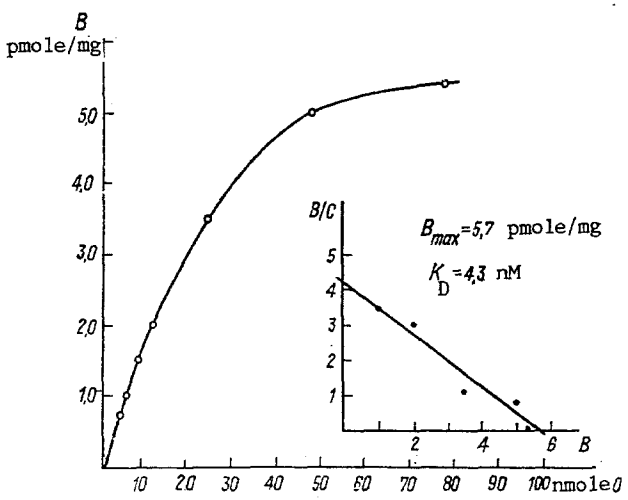

Fig. 1. Specific binding of ^{3}H-BAP with the CBP fraction eluted by 60% ethanol from a hydrophobic column of Plikhrom-1. In the inset, the graph is shown in the Scatchard coordinates.

fic sorbents. Therefore, to eliminate low-molecular-mass components the protein fraction obtained from the plant tissue homogenate was subjected to gel filtration on Sephadex G-25. To extract the CBP site, the eluate obtained was chromatographed in a 0-1 M gradient of NaCl on a biospecific sorbent containing a synthetic cytokinin – BAP – as ligand. The fraction eluted by 1 M NaCl was chromatographed further on the hydrophobic sorbent Polikhrom-1 with ethanol in a concentration gradient of from 0 to 96%.

For the analysis of the binding activity with ^{3}H BAP of the fractions obtained we used the method of sorption on Synpor membrane nitrocellulose filters (Table 1). The greatest binding activity was possessed by the fraction eluted by 60% ethanol. The parameters of its binding with ^{3}H BAP were characterized by the binding constant K_D = 4.3 nM, and the maximum amount of binding sections B_{max} = 5.7 pmole/mg of protein (Fig. 1). However, HPLC analysis (Fig. 2) showed that this fraction contained, in addition to the main cytokinin-binding protein, other, impurity, proteins.

Apparently, the proteins present in the CBP site have very close hydrophobic characteristics, and Polikhrom-1 is not completely suitable for the selective separation of proteins of this nature. For the further purification of the CBP, therefore, we developed a specific sorbent by modifying the commercial gel TSK-HW-65 with phenyl groups. This support, combining the properties of a molecular sieve with hydrophobicity proved to be a high-capacity and effective sorbent (Fig. 3A).

The results of the analysis of the receptor activity of the fraction obtained (Table 2) permitted the conclusion that the CBP was localized mainly in fractions 4 and 5.

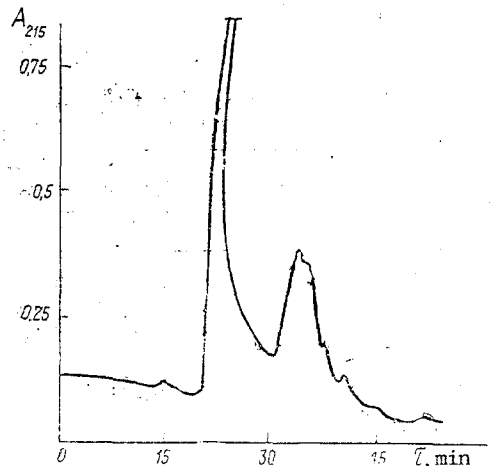

Fig. 2. HPLC analysis of the CBP fraction eluted by 60% ethanol from the hydrophobic sorbent Polikhrom 1.

In PAAG with SDS, fraction 4 migrated in the form of a single band with a molecular mass of about 43 kDa, while fraction 5, in addition to this protein, contained another two components, with molecular masses of 60 and 68 kDa, represented in the sixth fraction in isolated form. Since, as can be seen from Table 2, they did not possess binding activity, the high activity of the fifth fraction, twice that of the fourth fraction, can be explained by the assumption that these two components presumably form part of the protein composition of the receptor and intensify its cytokinin activity.

Thus, fraction 5 possibly reflects the true composition of the receptor complex or, at least part of it. The elucidation of the functions of each of these components requires further investigation.

EXPERIMENTAL

Isolation of a Fraction of Water-Soluble Proteins. Seeds of a cotton plant of variety 108-F were treated with concentrated sulfuric acid to remove fuzz, moistened for a day, and germinated on a moist porolon sponge for 2-3 days and they were then placed between sheets of filter paper. Moistening and germination of the seeds was carried out in the dark at 28°C. The seedlings freed from hulls were comminuted with knives and homogenized in a tissue mill (TR-1) in 8-10 volumes of 50 mM Tris buffer, pH 7.6, containing 50 mM KCl, 2.5 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, and 250 mM sucrose. The homogenate was filtered through a layer of coarse calico, and was centrifuged at 18,000 rpm for 30 min. This led to the precipitation of a fraction of coarse membranes. The water-soluble proteins present in the supernatant were precipitated with ammonium sulfate at 90%-saturat the mixture was kept in the cold, and the precipitate was collected by centrifugation at 18,000 rpm and was dialyzed.

Synthesis of Bap-Acetamidohexyl-Sepharose. The attachment of the BAP ligand was carried out by the alkylation of iodoacetamidohexyl-Sepharose. The reaction was carried out in dioxane in which 2.79 g of iodoacetic acid and 2.7 g of N-hydroxysuccinimide had been dissolved. The precipitate of dicyclohexylurea that deposited was eliminated by filtration. The filtrate was added to a suspension of aminohexyl-Sepharose, and the reaction was performed at 4°C. A 10^{-3} M solution of BAP was added to the iodoacetamide gel and the mixture was kept at room temperature for three days. The sorbent obtained contained 10 μmole of BAP per 1 ml of gel.

Affinity Chromatography. The protein fraction issuing with the free volume of a column of Sephadex G-25 was deposited on the BAP-acetamidohexyl-Sepharose gel. The column was washed with the buffer Tris-HCl 50 mM, pH 7.6, until an eluate with a constant optical density at 208 nm was obtained. The bound proteins were eluted by 1 M NaCl at the rate of 0.3 ml/min. The protein fraction was dialyzed against water, freeze-dried, and stored at −4°C. Amounts of protein were determined by Lowry's method [5].

High-Performance Liquid Chromatography (HPLC). HPLC was conducted on an Ultrapac TSK-3000 column. The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.6, buffer. The

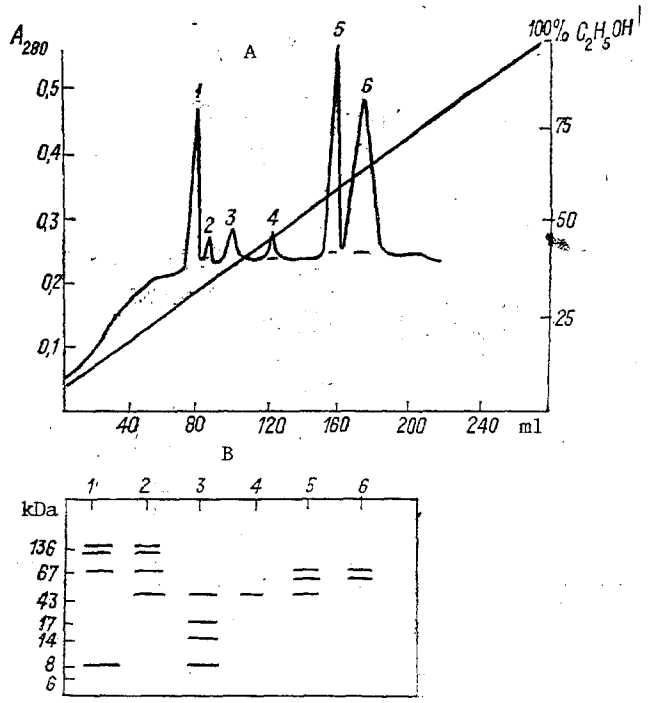

Fig. 3. Chromatographic separation of the CBP site on a column of TS-HW-65-F (A), and electrophoretic analysis of the fractions obtained (B).

protein was deposited on the column, and the column was washed with the same buffer. The absorption at 280 nm was recorded. Marker proteins were calibrated in individual experiments.

Electrophoresis was performed in a gradient (from 9 to 25%) PAAG gel containing sodium dodecyl sulfate [6].

Purification of the CBP on a Hydrophobic Column of Polikhrom-1. Purification was carried out on a column (1 × 10 cm) of the sorbent Polikhrom (Reakhim). The fraction eluted by 1 M NaCl was deposited on the hydrophobic column, and the non-specifically-bound proteins were washed out with water. Elution was performed with a stepwise gradient of ethanol (20, 40, 60, 80, and 96%). Proteins were detected on a Uvicord instrument at 280 nm.

Purification of the Proteins on a Hydrophobic Column with TSK-HW-65. The fraction eluted by 1 M NaCl was deposited on the column equilibrated with 1 M NaCl. The column was equilibrated with a reversed gradient (1/M NaCl—water). Proteins were eluted with a linear gradient of ethanol.

Experiments on the Binding of ^{3}H-BAP. The method of filtration on Synpor nitrocellulose filters (Czechoslovakia) was used. ^{3}H-BAP with a specific activity of 24 Ci/mmole was charged into micro-test-tubes. The concentration of added ^{3}H-BAP was selected so that the final volume of 100 µl contained the required amounts of labeled BAP (from 10^{-9} to 10^{-6} mole). Then to each tube was added 10 µl of water, or, in the control, 10 µl of a solution of unlabeled BAP in a concentration of 10^{-2} M for determining the level of nonspecific binding. After this, 80 µl of the preparation of proteins in buffer for binding was added.

Binding with the preparation of membrane proteins was carried out in 10 mM sodium citrate, pH 5.5, and with the water-soluble fractions in 25 mM Tris-HCl, pH 7.6. Each mixture was incubated at 20°C for 20-40 min. After the end of incubation, 80 µl of the mixture was deposited, with the vacuum switched off, on the mat side of a filter placed in a filter funnel and previously wetted with distilled water. Then the vacuum was switched on, the mixture was sucked off, and the filter was washed with 5 ml of water. After this, the filters were dried, placed in bottles, and covered with 3 ml of standard toluene scintillator. Radioactivities were determined on a Pac Beta counter (LKB, Sweden).

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ELECTROHYDRAULIC EFFECT IN WATER-PLANT HETERODISPERSE SYSTEMS

I. COMPOSITIONS OF THE SOLID RESIDUE AND THE AQUEOUS PHASE

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The electrohydraulic (EH) treatment of water-plant heterodisperse systems leads to the partial degradation of the main components of the plant materials: lignin, cellulose, and carbohydrates. The presence of p-hydroxyphenyl and guaiacyl structures has been established on the basis of the results of the GLC analysis of the ether-extracted phenolic products of plant materials subjected to EH treatment. It has been shown that part of the carbohydrates passes into the aqueous phase in the form of free monosaccharides: pentoses and hexoses.

Electrohydraulic shock (EHS) – a high-voltage spark discharge in a liquid at a potential difference between the electrodes of 10-100 kV with a current amplitude of 1-50 mA – is capable of causing mechanochemical reactions with the participation of the components of the medium [1, 2]. The discharge is accompanied by the appearance of a branched system of streamers, the generation of powerful shocks and acoustic effects, and other phenomena, leading to the activation of the heterodisperse system in the interelectrode space.

There is only a little information in the literature on the practical application of this effect [2-4]. EHS cannot be included among purely mechanical effects: it is a multifactorial physicochemical process in which shock waves having a hydrostatic pressure of 10^2-10^3 MPa, plasma with a temperature of 10^4 K, a pulsed electric field, and x-radiation and luminous and thermal radiation all participate.

Hitherto, many physicochemical aspects of electrohydraulic processes have remained little studied. Having in view the highly promising possibilities of the electrohydraulic effect (EHE) in both the theoretical and the applied respects, we have investigated water-plant heterodisperse systems subjected to treatment by the EHE with the aim of elucidating

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