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ISOLATION FROM COTTON SHOOTS OF AN AUXIN-BINDING PROTEIN REGULATING THE SYNTHESIS OF RNA

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With the aid of a complex method of purification, including affinity chromatography and HPLC, from a fraction of the water-soluble protons of cotton seeds we have isolated a protein which binds the labeled auxin [^{3}H]IAA with high affinity (K_d = 6 nM) and in the presence of IAA stimulates the synthesis of RNA in vitro *in a preparation of cottonplant chromatin. In PAAG electrophoresis with sodium dodecyl sulfate, this protein gave a single band corresponding to a molecular mass of 43 kDa. It is assumed that this protein is a receptor for the auxin phytohormone that is responsible for regulating gene activity.*

According to current ideas, the action of an auxin plant hormone at the cell level is mediated by its binding with specific receptors. On the basis of information in the literature on auxin-binding proteins (ABPs) it is possible to assume the possibility of the existence in cells of several types of receptors for an auxin that are responsible for the manifestation of its action both at the membrane level and at the level of the synthesis of RNA and protein [1]. In membrane preparations from various species of plants proteins have been detected that bind auxins with a high affinity in an acid medium (pH 5-6). On the basis of the results of a number of experiments, it may be assumed that some of these proteins are auxin receptors regulating the growth of cells by dilatation.

In the cytoplasm and nuclei of cell have been found nonmembrane ABPs with a high affinity for an auxin — indol-3-ylacetic acid (IAA) — (K_d = 1-10 nM) in a neutral or weakly alkaline medium (pH 7-8) which are possibly another type of auxin receptors, since in *in vitro* experiments in the presence of auxins they activate the synthesis of RNA in the nuclei [2-4]. These nonmembrane or soluble ABPs have been best characterized for cultivated tobacco cells [5]. They each have a molecular mass of 150-200 kDa and consist of 3-4 subunits with molecular masses of about 50 kDa. The auxin-binding activity of these proteins correlated positively with the degree of their phosphorylation.

When supplies of auxins in a culture liquid are depleted, a soluble auxin-binding protein accumulates in the cytoplasm, and on the addition of a fresh portion of auxins a migration of this protein into the nucleus is observed and, in parallel with this, the nuclear synthesis of RNA is activated. In an investigation of the soluble ABPs from the cotton plant, IAA-binding proteins were detected in the fraction of acidic nonhistone proteins from cotton shoots that stimulated the nuclear synthesis of RNA in the presence of an auxin [6].

We have previously [7] found auxin-binding sites both in membrane fractions and in fractions of soluble proteins from cotton shoots. In the present paper we consider the isolation and the results of a study of the properties of cottonplant ABPs taking part in the regulation of gene activity.

To isolate the total ABPs, we used affinity chromatography. On the basis of the results of a study of the structures of compounds possessing an auxin-like action the idea arose that for binding with auxin receptors the auxin analogs should have a free carboxy group [8]. We therefore synthesized a sorbent containing IAA as ligand, which was added through an imino group to the "spacer" AH-Sepharose 4B. The extract of water-soluble proteins was first desalted by gel filtration on Sephadex G-25 and was then passed through a column with IAA-acetamidohexyl-Sepharose. The bound proteins were eluted with 1 M NaCl. The total amount of protein eluted from the column, evaluated from the area of the peak, was 9%.

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TABLE 1. Inclusion of [^{3}H]UTP in a Nuclear Preparation of Chromatin Enriched with RNA Polymerases from Cotton Shoots in the Presence of the ABP Purified by Affinity Chromatography

Experiment	Inclusion of [^{3}H]UTP in the nuclear chromatin	
	Pulses/min	%
Control	6092 ± 920	100
IAA	6992 ± 804	115
ABP	5421 ± 842	89
ABP+IAA	10040 ± 883	178

TABLE 2. Inclusion of [^{3}H]UTP in a Chromatin Preparation in the Presence of IAA and the Fractions Obtained with the Aid of HPLC

Fractions	Inclusion of [^{3}H]UTP	
	Pulses/min	%
Control	3567 ± 223	100
IAA	4322 ± 294	121
200 kDa+ IAA	7106 ± 351	191
50 kDa+ IAA	4348 ± 235	122
30 kDa+ IAA	4520 ± 287	126
15 kDa+ IAA	4487 ± 234	125
8 kDa + IAA	4308 ± 293	120

After the desalting of the eluate, we studied the specific binding of [^{3}H]IAA in it. The concentration of IAA-binding sites measured at a concentration of [^{3}A]IAA of 30 nM in the fraction of protein deposited on the affinity column amounted to 0.8 pmole/mg of protein, while in the eluate from the affinity column it was 20 pmole/mg of protein.

Thus, the purification of the ABPs with the aid of affinity chromatography amounted to not less than 25-fold. It must be mentioned that the concentration of IAA-binding sites in the protein fraction after affinity chromatography was most probably underestimated, since in the experiments on binding it was difficult to determine the amount of protein remaining on the filters when only small amounts of protein (less than 10 μg) were deposited on them.

As can be seen from Table 1, the ABP fraction purified by affinity chromatography possessed a capacity for activating the synthesis of RNA in *in vitro* experiments. When this fraction was stored in the freeze-dried state at $-10°C$ for several months, it retained both its auxin-binding activity and its capacity for stimulating the synthesis of RNA.

Electrophoresis in PAAG with sodium dodecyl sulfate (SDS) of the proteins eluted from the affinity column is represented by a densitogram (Fig. 1) from which it can be seen that the eluate contained a large number of individual proteins.

The further purification and the estimation of the molecular masses of the ABPs were carried out by gel filtration on an LKB Glas Pac TSK HW-3000 column (8 × 300 mm) with the aid of an HPLC apparatus. The elution profile is shown in Fig. 2. The eluate consisted of components with molecular masses of from 200 to 8 kDa.

Five main peaks were selected, and each of them was investigated for binding with [^{3}H]IAA and capacity for stimulating the inclusion of [^{3}H]UTP in chromatin. It was found that this capacity was possessed by the peak with a molecular mass of 200 kDa (Table 2). This protein specifically bound [^{3}H]IAA (30 nM) with a concentration of binding sites of not less than 150 pmole/mg of protein.

If we start from the assumption that one molecule of IAA should be bound with each molecule of an ABP having a molecular mass of 200 kDa, then, theoretically, the concentration of binding sites should be 5000 pmole/mg of protein. In our experiments, no values higher than 200 pmole/mg of protein were obtained. An accurate determination of the number of binding sites is made difficult by the fact that binding was studied in an eluate with a very low (less than 1 μg/ml), and therefore undetermined, concentration of protein. The amount of protein with a molecular mass of 200 kDa evaluated from the area of the peak (Fig. 2) was about 13% of the total proteins separated in HPLC.

Figure 3 shows the curve of the specific binding of [^{3}H]IAA with a preparation of the water-soluble fraction obtained in the range of concentrations of labeled auxin of from 1 to 100 nM. The graph in the Scatchard coordinates shows K_d = 6 nM.

To evaluate the subunit composition of the purified ABP with a molecular mass of 200 kDa we performed electrophoresis under denaturing conditions. The results are shown in the form of a densitogram (Fig. 4), from which it can be seen that the ABP consisted of 43-kDa subunits. Apparently the protein that we had isolated with a molecular mass of about

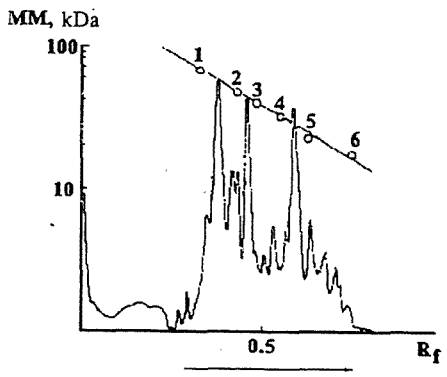

Fig. 1

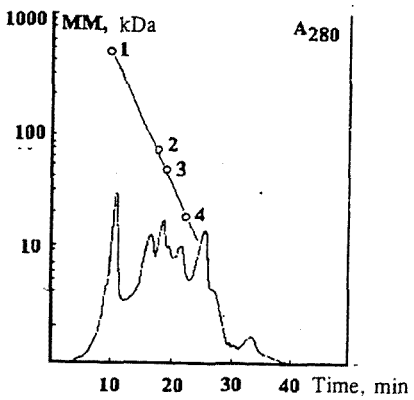

Fig. 2

Fig. 1. Densitogram of the results of electrophoresis in PAAG with SDS of the eluate from the affinity column. Marker proteins: 1) bovine albumin (68 kDa); 2) egg albumin (45 kDa); 3) carboxyanhydrase (29 kDa); 4) trypsin inhibitor (20.1 kDa); 5) myoglobin (17.8 kDa); 6) cytochrome C (12.3 kDa).

Fig. 2. HPLC of the ABPs eluted fom the affinity column. Marker proteins: 1) ferritin (450 kDa); 2) bovine albumin (68 kDa); 3) ovalbumin (43 kDa); 4) myoglobin (17.8 kDa).

200 kDa from the cotton plant, just like a protein with a molecular mass of 200 kDa from a culture of tobacco cells [9] and a protein with a molecular mass of 190 kDa from kidney beans [10], is an auxin receptor regulating gene activity.

Thus the results obtained permit the development of investigations in the direction of determining the synthesis of which mRNAs and the proteins corresponding to them are activated by a soluble ABP and also the mechanism of this activation. The methods developed for isolating ABPs also permit the development of another direction of practical importance — the primary screening of new plant growth regulators.

EXPERIMENTAL

We used hypocotyls of shoots of the cotton plant *Gossypium hirsutum* L., variety 175-F. The seeds were treated with sulfuric acid to remove the fuzz, and were then steeped in water for 20 h, and germinated in the dark on moist filter paper at 28°C for three days.

The hypocotyls were comminuted with knives and homogenized in 10 volumes of a solution containing 50 mM Tris-HCl, pH 7.8, 50 mM KCl, 2.5 $MgCl_2$, 5 mM EDTA, 2 mM DTT, and 250 mM sucrose, first in a tissue grinder, and then in a glass/PTFE homogenizer with five passes of the pestle at 1000 rpm. The homogenate was filtered through a layer of coarse calico and was centrifuged at 100,000*g* for 60 min.

The supernatant, containing the water-soluble proteins, was 80% saturated with dry ammonium sulfate, the mixture was kept in the cold, and the precipitate was collected by centrifuging at 20,000*g* for 20 min. It was redissolved in a small volume of buffer (50 mM Tris-HCl, pH 7.8), and the insoluble particles were removed by centrifugation. For desalting and the elimination of low-molecular-mass substances, the protein fraction was passed through a column of Sephadex G-25 (2.6 × 50 mM) equilibrated with the buffer, and elution was carried out at the rate of 30 ml/h. For the subsequent work we used the protein fraction issuing in the free volume of the column.

To obtain an ABP fraction, the protein fraction was deposited on a column (2.6 × 10 cm) of IAA-acetamidohexyl-Sepharose, and this was washed with the buffer 50 mM tris-HCl, pH 7.8, until an eluate with a constant optical density at 280 nm was obtained. The bound proteins were eluted with 1 M NaCl at the rate of 3 ml/min. The ABP fraction was dialyzed against water, freeze-dried, and stored at −10°C.

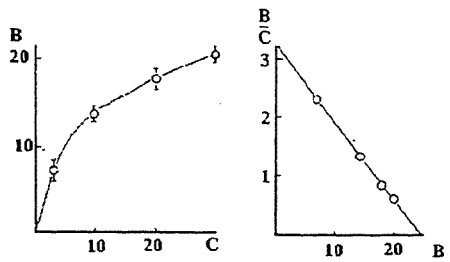

Fig. 3

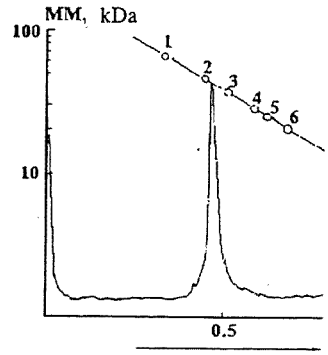

Fig. 4

Fig. 3. Binding of [^{3}H]IAA with an ABP after HPLC: 1) binding in direct coordinates; 2) in the Scatchard coordinates; B) amount of [^{3}H]IAA bound, pmole/mg of protein; C) concentration of [^{3}H]IAA, nM.

Fig. 4. Densitogram of the results of electrophoresis in PAAG with SDS of a protein having an apparent molecular mass of 200 kDa evaluated by HPLC. Marker proteins the same as in Fig. 1.

The HPLC of the ABPs was carried out in an LKB (Sweden) Glas Pac TSK HW-3000 column (8 × 300 mm). The column was equilibrated with a 0.1 M K_2HPO_4/KH_2PO_4, pH 6.8, buffer, containing 0.1 M NaCl, and then 100 μl of a 10 mg/ml solution of the protein in the buffer was deposited on a column and chromatographed in the same buffer at a rate of flow of 0.5 ml/min under a pressure of 20 bar. The absorption at 280 nm was recorded. In individual experiments, to evaluate the molecular masses of the ABP fractions, the column was calibrated with marker proteins.

Electrophoresis of the proteins in the presence of SDS was carried out in a gradient ofpolyacrylamide gel (5-20%) [11].

Protein was determined by staining with Coomassie G-250 [12], ovalbumin being used as standard.

The experiments on binding were carried out by filtration on Synpor nitrocellulose filters (Czechoslovakia) with pore dimensions of 0.4-0.8 μm, as described previously [7].

RNA polymerase activity was determined as described in [13].

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