Chemistry of Natural Compounds, Vol. 34, No. 5, 1998

INFLUENCE OF GIBBERELLIN-BINDING PROTEINS OF THE COTTON PLANT ON THE FUNCTIONING OF THE GENOME

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UDC 577.112.083

Gibberellin-binding proteins have been isolated from cotton plant seedlings with the aid of a Sepharose-GA $_3$ affinity column. The proteins obtained have been characterized electrophoretically and their action in isolated nuclei and transcription-active chromatin has been studied in vitro.

The most important bioregulators are hormones, which control an enormous number of intracellular processes. Among plant hormones the gibberellins occupy a special place [1]. They participate in the stimulation of growth and flowering; the synthesis and technological qualities of cotton fiber are, in the main, controlled by a gibberellin [2, 3]. It is known that the action of the gibberellin is effected through the formation of a hormone-receptor complex. The isolation of the gibberellin receptor and the elucidation of the mechanism of its action are therefore important for the genetic engineering of plant cells.

We have isolated gibberellin-binding proteins (GBPs) and have studied their role in the transcription of chromatin.

Two-day cotton plant seedlings were homogenized in 5 mM tris-HCl buffer, pH 7.2, containing 0.14 M NaCl, and the homogenate was centrifuged. The supernatant obtained after centrifuging was subjected to gel filtration on a column of Bio-Gel A6 and the fraction containing proteins with molecular masses of less than 400 kDa was collected. Further fractionation of the protein was carried out on an affinity column of Sepharose 4B—gibberellin. To obtain the affinity column we used gibberellic acid GA_3, since the function of this compound has been investigated to the greatest extent in plants, including the cotton plant [4].

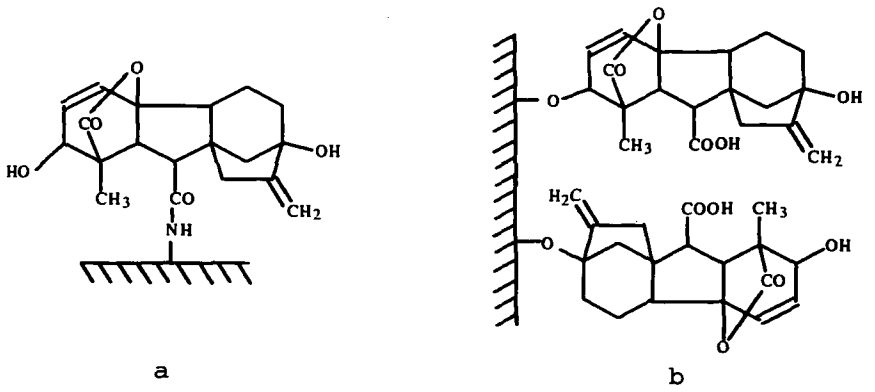

Fig. 1. Structures of affinity sorbents with gibberellic acid as ligand.

We synthesized two sorbents. In the first sorbent the GA_3 was attached to aminohexyl-Sepharose with the aid of carbodiimide. In this case, the ligand was attached by an amide bond formed by the COOH group at C-7 and an NH_2 group of

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0009-3130/98/3405-0631$20.00

the matrix (Fig. 1, *a*). In the second case, the GA_3 was added to epoxy-activated Sepharose and the ligand was attached through an ether bond formed by the OH group at C-3 or that at C-13 and the oxirane group of the matrix (Fig. 1 *b*). The structure of the ligands in the affinity sorbents used resembles the structure of gibberellin conjugates [5, 6], for which a possibility of interaction with a receptor is unknown. After affinity chromatography, therefore, we studied the compositions of the proteins bound to each of the sorbents separately.

The bound proteins were separated by stepwise elution with 0.1 M NaCl—0.6 M NaCl and 0.8 M NaCl—50% ethylene glycol. The elution profiles of the proteins bound with the GA_3-Sepharose were identical for the two types of sorbents (Fig. 2).

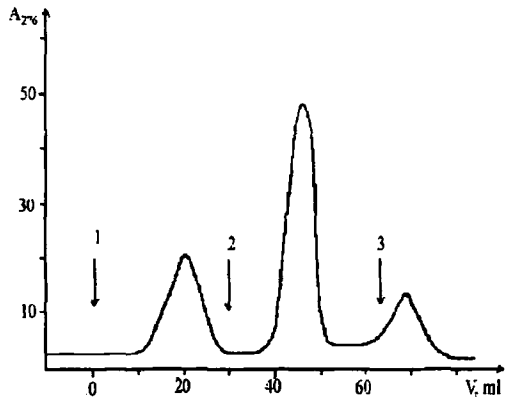

Fig. 2. Affinity chromatography of soluble proteins from cotton seedlings on GA_3-Sepharose (1 × 10 cm): peak *1*) elution with 0.1 M NaCl (GBP-1); *2*) 0.6 M NaCl (GBP-2); *3*) 0.8 M NaCl with 50% polyethyleneglycol (GBP-3).

The proteins obtained were characterized electrophoretically [7] (Fig. 3). Electrophoretic analysis showed a heterogeneous composition of the GBPs, while the compositions of the proteins in the eluates from the GA_3-Sepharoses of both types were identical. However, when we compared the composition of the eluate obtained by salt elution (0.1 and 0.6 M NaCl) with that of the eluate under the conditions of regeneration of the sorbent (0.8 M NaCl with 50% of polyethyleneglycol) differences were found only in the ratio of certain fractions. The fraction obtained under the sorbent-regenerating conditions (0.8 M NaCl with 50% of polyethyleneglycol) was the least heterogeneous. The heterogeneity of the fractions obtained can be explained by the fact that the seedlings were rich in enzymes of the metabolism of the gibberellin and in proteins responsible for its transport and for the regulation of hormonal activity. For example, a considerable part of the gibberellin in seeds is present in the form of inactive conjugates which, on being enzymatically hydrolyzed during germination, form the free, physiologically active, gibberellic acid [8].

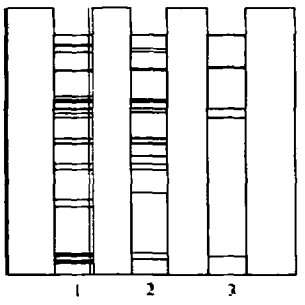

Fig. 3. Electrophoregrams of the GBPs obtained by affinity chromatography on GA_3-Sepharose (10% PAAG—Na-SDS): *1*) 0.1 M NaCl eluate; *2*) 0.6 M NaCl; *3*) 0.8 M NaCl with 50% of polyethyleneglycol.

It is known that many of the effects of a gibberellin are due to its influence on the activity of a group of genes. In view of this, we have studied the action of GBPs on the transcriptional activity of the genome. Seeds were germinated in the presence of 10^{-5} M gibberellin A_3 for two days. Nuclei were isolated form the two-day seedlings as described in [9]. As control we used nuclei isolated from two-day seedlings grown without the addition of a gibberellin. Assuming that the isolated nuclei contained their own RNA polymerase, we studied the synthesis of RNA in the isolated nuclei from the inclusion of ^{3}H-UTP, using an incubation medium for the synthesis of RNA. It was found that the inclusion of ^{3}H-UTP (synthesis of RNA) rose by 70—80% in the experimental samples as compared with the control.

In the same system we studied the action of various fractions of the GBPs isolated by means of the Sepharose—gibberellin affinity column (Table 1). As can be seen from the table, only the fraction of GA_3-binding proteins

eluted by 0.6 M NaCl exhibited a most appreciable stimulating effect on the inclusion of the labeled precursor in the NA. The protein with the properties of a gibberellin receptor was obviously present in the fraction eluted from the GA_3- Sepharose column by 0.6 M NaCl. We subsequently studied the action of this protein fraction on transcription-active sections of chromatin.

TABLE 1. Influence of GBPs on the Synthesis of NA in Isolated Nuclei of Cotton Plant Seedlings

Experiment No.	Fraction	Inclusion of ^{3}H-UTP, %
1	N (control)	100
2	N + GBP	110
3	N + GBP-1	100
4	N + GBP-2	140
5	N + GBP-3	90

Note: N — nuclei; GBP — total fraction; GBP-1 (0.1 M NaCl eluate); GBP-2 (0.6 M NaCl eluate); GBP-3 (0.8 M NaCl eluate with 50% of ethylene glycol). Each sample contained 100 μg of nuclei and 50 μg of GBP.

Transcription-active chromatin was obtained from nuclei [9] isolated from two-day cotton seedlings. To obtain the soluble (transcription-active) chromatin, the nuclei were treated with micrococcal nuclease in a proportion of 1 μg of nuclease to 1 mg of DNA for 40 s (see the Experimental part). This regime was the optimum, since for studying transcriptional activity it is important to obtain polynucleosomes. The regime for nuclease treatment was selected on the basis of an electrophoretic analysis of the DNP. For the lysis of the nuclei and to obtain transcription-active chromatin after treatment with the nuclease, the nuclear material was dialyzed overnight against 0.2 mM EDTA and was then centrifuged, and the supernatant, which contained the transcription-active chromatin was used for further work [10, 11].

To study the transcription of chromatin under the action of various fractions of the GBPs we isolated RNA polymerase from *E. coli* by the method of Parker et al. [12] with some modification. RNA polymerase is used in the study of the regulation of the genome at the level of transcription in eukaryotic cells since both bacterial and homologous RNA polymerases are capable of recognizing the "active" sections of chromatin and performing transcription in them; i.e., transcription in vitro reflects the pattern of the tissue-nonspecific expression of genes in vitro [13, 14]. The synthesis of RNA was studied from the inclusion of ^{3}H-UTP (Table 2).

TABLE 2. Action of GBPs on the Transcriptional Activity of Chromatin

Experiment No.	Sample	Inclusion of ^{3}H-UTP, %
1	Chr (control)	100
2	Chr + G	126
3	Chr + GBP-1	95
4	Chr + GBP-2	136
5	Chr + GBP-3	90
6	Chr + G + GBP-1	116
7	Chr + G + GBP-2	167
8	Chr + G + GBP-3	116

Note: Chr — chromatin, 10 μg; G — gibberellin GA_3, 0.05 μg; GBP — 5 μg.

The greatest stimulating effect on transcription-active chromatin was exhibited by the GBP fraction eluted from the affinity column by 0.6 M NaCl (GBP-2), this effect being most pronounced on the addition of gibberellin to the sample. Some stimulation of transcription was observed when gibberellin (No. 2) and GBP-2 (No. 4) were added to the sample. It is possible that in transcription-active chromatin there are sites of binding both with the hormone and with protein, which explains this effect. GBP was also isolated by affinity chromatography using GA_3 as ligand. As can be seen from electrophoregrams, the

proteins were insufficiently purified and contained as impurities other proteins that may possibly make their own contribution to transcription. Nevertheless, numerous experiments conducted on animal cells, in particular on the investigation of thyroid hormones, show that the hormone-receptor complex is localized mainly at the boundary between the hetero- and the euchromatin [15—17], which ensures its definite participation in the regulation of the transcriptional activity of the genome.

We also studied the influence of concentration on the transcriptional activity of the genome. For this, chromatin, protein, and hormone were added to the reaction mixture in concentrations 5 times lower than in the initial mixture (Table 3). No appreciable differences were observed in the stimulation of transcription. The results obtained permit the assumption that a specific interaction takes place at definite concentrations that leads to the stimulation of transcription and a further increase in concentration has no appreciable effect.

TABLE 3. Transcriptional Activity of a GBP

Experiment No.	Sample	Inclusion of ^{3}H-UTP, %
1	Chr (control)	100
2	Chr + G	119
3	Chr + GBP-2	143
4	Chr + G + GBP-2	179

Note: Chr — chromatin, 2 μg; G — 0.01 μg; GBP — 1 μg.

Thus, it has been shown on cotton plant seedlings that transcription-active chromatin contains sites recognizing a gibberellin—GBP-2 complex the formation of which leads to a stimulation of transcription, while when the concentration is increased no appreciable changes in template activity are observed.

EXPERIMENTAL

The GBP was isolated from two-day cotton plant seedlings. The seedlings were homogenized in 50 mM tris-HCl buffer, pH 7.2, containing 0.14 M NaCl, 5 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was filtered through four layers of gauze and the filtrate was treated with about 1% of Tween-20, after which NaCl was added to a final concentration of 1 M. The homogenate was centrifuged on a Beckman J-2-21 centrifuge at 20,000 rpm for 1 h. The supernatant was subjected to gel filtration on a 4 × 8 cm column of Bio-Gel A-6 in 50 mM tris-HCl, pH 7.2, containing 5 mM EDTA, 1 M NaCl, and 3 μmole of PMSF at a rate of elution of 30 ml/h. The column was calibrated with high-molecular-mass spleen DNA (free volume), Blue Dextran-2000 (2×10^6 kDa), immunoglobulin (160 kDa) and cytochrome C (12.4 kDa).

Fractions of the eluate corresponding to proteins with molecular masses of less than 400 were collected. The total soluble proteins obtained in this way were dialyzed against 20 mM K phosphate buffer, pH 6.8, containing 1 mM EDTA. Then these proteins were deposited on an affinity column (1 × 4 cm) with gibberellin-Sepharose. The affinity column was equilibrated with 20 mM K phosphate buffer containing 50 mM NaCl, and stepwise elution of the bound proteins was conducted with 0.1, 0.6, and 0.8 M NaCl containing 50% of ethylene glycol. The fractions collected were dialyzed, lyophilized, and used for the subsequent investigations.

To obtain the affinity column with gibberellin, a solution of 100 mg of Serva gibberellic acid (GA_3) in 1 ml of dioxane was diluted to 10 ml with 0.1 M Na carbonate buffer, pH 8.0. Epoxy-activated Sepharose 4B from Pharmacia (3 g) was washed with 200 ml of water and was stirred with the solution of GA_3 in carbonate buffer. Binding was achieved by the continuous stirring of the suspension at room temperature for 18 h. The amount of GA_3 bound was determined from the difference between the amount of gibberellin in the initial solution and the amount of nonbound gibberellin after the gel had been washed. The epoxy groups that had remained free were blocked by the action of a 1 M solution of ethanolamine at room temperature for 1 h.

Nuclei were isolated from two-day seedlings as described in [9]. The purity of the nuclei obtained was checked microscopically.

Transcription-active chromatin was isolated from the nuclei by treatment with micrococcal nuclease [10]. For this, the

nuclei were transferred into a solution containing 10 mM tris-HCl, pH 7.9, and 0.1 mM $CaCl_2$ previously heated to 37°C, micrococcal nuclease was added in a proportion of 1 μg per 1 mg of DNA, and the mixture was incubated for 1.5 h at 37°C. An aliquot was taken for DNP-phoresis every 10 s. The reaction was stopped by placing the solution in ice and adding EDTA to a final concentration of 0.2 mM. It was then dialyzed against 0.2 mM EDTA overnight. After dialysis the solution was centrifuged at 15 thousand rpm on a VAC centrifuge for 30 min. The deposit was removed, and the supernatant, containing the soluble chromatin, was used for further work.

DNP-phoresis was conducted as described in [10]. The initial gel contained acrylamide (28%) and methylene-bisacrylamide (0.75%). The working gel contained 7% PAAG, 2 mM EDTA, and 10 mM tris-HCl, pH 8.3. Composition of the buffer: 10 mM tris-HCl, pH 8.3, and 2 mM EDTA. The gel was stained with ethidium bromide and the bands of the relevant nucleosomes were viewed under ultraviolet illumination.

The incubation mixture for determining RNA polymerase activity had the following composition: 40 mM tris-HCl, pH 7.9—10 mM $MgCl_2$—0.1 mM EDTA—0.1 M DTT (dithiothreitol)—0.2 M KCl—0.05% BSA—0.2 mM of each of the four nucleoside triphosphates (ATP, GTP, CTP, UTP), and the labeled ^{3}H-UTP with an activity of 40 MBq.

Various concentrations of chromatin, GBP, hormone, and RNA polymerase in a ratio of DNA to enzyme of 1:2 were added to the incubation mixture (100 μl) and the samples were incubated at 37°C for 15 min. After the end of the reaction, the samples were cooled, a 5% solution of TCA was added, and the mixtures were transferred to GFC filters. The filters were washed three times with a cold 5% solution of TCA and then with 96% alcohol and were dried in the air and counted in 5 ml of scintillator (PPO—POPOP in toluene) in a LS-230 liquid scintillation counter (Beckman). The level of synthesis of RNA was judged from the inclusion of ^{3}H-UTP in the acid-insoluble fraction.

The proteins obtained were characterized in 10% PAAG in the presence of 0.1% sodium dodecyl sulfate [7].

Protein contents were determined by the Lowry method [18].

DNA concentrations were measured spectrophotometrically on the basis of the relation $A_{260nm} = 20$ units for 1 mg of DNA, or by Spirin's method [19].

All operations for the isolation of protein, nuclei, and soluble chromatin were conducted at 4°C.

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