Chemistry of Natural Compounds, Vol. 30, No. 1, 1994

SYNTHESIS OF [^{3}H]ABSCISIC ACID AND IDENTIFICATION OF THE ABA-BOUND PROTEINS OF THE COTTON PLANT

N. Zh. Sagdiev, O. N. Veshkurova, A. A. Takanaev, Zh. F. Ziyavitdinov, Sh. Ya. Mirzaakhmedov, U. K. Inogamov, and S. I. Salikhov

UDC 547.857.581.192,7

[^{3}H]Abscisic acid has been obtained by heterogeneous catalytic exchange in a tritium-containing solvent. The presence in cottonplant shoots of proteins specifically binding with abscisic acid has been shown.

The isolation from cottonplant shoots of proteins exhibiting a high affinity for auxins, cytokinins, gibberellins, and ethylene has been reported previously [1-4]. Some of their physicochemical characteristics and functional activities have been investigated. A continuation of these investigations is the isolation of cottonplant proteins specifically binding abscisic acid (ABA). As our investigations have shown, the content of hormone-binding proteins in the cotton plant is very low and their identification requires radioactively labeled phytohormones with a high specific activity. With this aim, we have developed a method of obtaining [^{3}H]ABA with a high specific activity by heterogeneous catalytic exchange in a tritium-containing solvent.

The total water-soluble proteins of four-day cottonplant shoots were freed from low-molecular-mass impurities by precipitation with ammonium sulfate. As was shown by the results obtained, ammonium sulfate at 90% saturation precipitated the proteins from the supernatant practically completely, and a further rise in the salt concentration did not lead to an increase in the yield of protein. The precipitate was then redissolved in a buffer containing 0.05 M Tris-HCl, pH 7.6, and was deposited on a 2.6 × 70 cm column of Sephadex G-50. Only one protein-containing fraction was obtained. This fraction was deposited on a column containing an affinity sorbent consisting of ABA conjugated with epoxy-Sepharose. The elution profile and the conditions of separation are shown in Fig. 1. The fraction eluted by 1 M NaCl (C_2) exhibited the greatest binding activity in experiments with]^{3}H]ABA.

After desalting and freeze-drying, the fraction obtained was analyzed by electrophoresis in PAAG (Fig. 2). As can be seen from Fig. 2, this fraction contained three proteins with molecualr masses of from 20 to 70 kDa. The further separation of fraction C_2 was achieved by hydrophobic chromatography on the sorbent Polikhrom-1 (Fig. 3).

After chromatography in an isopropanol gradient, three fractions were obtained, of which the greatest affinity for [^{3}H]ABA was possessed by fraction D_3 As the results of electrophoresis showed, fraction D_3 was represented by a single protein band with a molecular mass of ~48 kDa (see Fig. 2).

Thus from the fracxtion of water-soluble proteins of the cotton plant we had isolated a highly purified protein exhibiting affinity for [^{3}H]ABA.

EXPERIMENTAL

Synthesis of [^{3}H]ABA. A solution of 50 mg of ABA in 0.5 ml of mrthanol was treated with 1 ml of high-percentage tritium water with a total activity of 10 TBq and 50 mg of Adams catalyst (PtO_2) and the mixture was heated in a sealed tube at 95-100°C for 6 h. After the end of the reaction the catalyst was removed by centrifugation. The labile tritium was eliminated by distillation with ethanol in vacuum. The [^{3}H]ABA was separated from radiolysis products by HPLC as in [5]. This gave 5.2 mg of [^{3}H]ABA with a molar activity of 2.8 GBq/mmole.

A. S. Sadykov Institute of Bioorganic Chemistry Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax 697071. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 109-112, January-February, 1994. Original article submitted July 13, 1993.

0009-3130/94/3001-0101$12.50

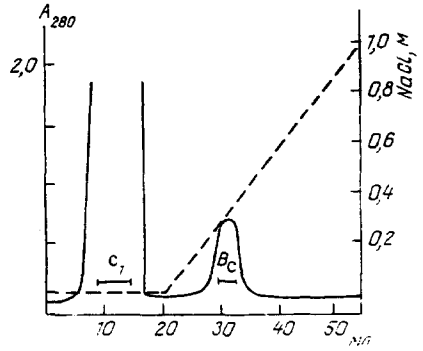

Fig. 1. Affinity chromatography of the protein fraction obtained after gel filtration on Sephadex G-50 in a column (1 × 10 cm) with agarose 6-ABA in 0.05 M TrisHCl, pH 6.0. Rate of flow 10 ml/h.

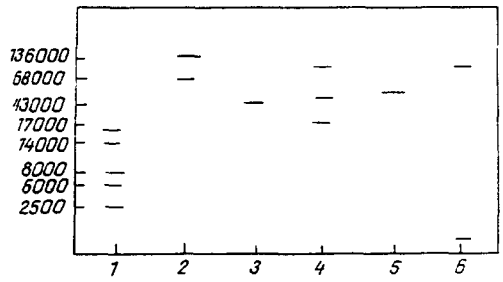

Fig. 2. Electrophoretograms obtained during the process of separating the proteins from cottonplant shoots: 1) BrCN-hydrolysate of myoglobin; 2) BSA; 3) ovalbumin; 4) fraction C_2 – affinity-soluble; 5) fraction D_3 — homogeneous hydrophobic soluble from B_2; 6) fraction E_2 — solubilized membranes after affinity chromatography.

Isolation of the Fraction of Water-soluble Proteins. Seeds of a cotton plant of the F-1 variety were treated with sulfuric acid to eliminate fibers and to sterilize them, and were washed with water to pH 7, after which they were grown in a thermostat at 27°C for 4-5 days. For the investigation we used young leaves of a four-day-old plant. In order to eliminate mesophilic cells, the leaves were placed in buffer with constant stirring at 4°C for 20 h. The standard buffer contained 50 mM Tris-HCl, 0.25 M sucrose, 2 mM mercaptoethanol, and 10 mM ascorbic acid, pH 7.5. Then the leaves were homogenized in the standard buffer at 8000 rpm in a Potter homogenizer. All the operations of subcellular fractionation were conducted at 4°C. The homogenate was filtered through three layers of gauze and was centrifuged at 48,000 *g* for 30 min. The supernatant was salted out with ammonium sulfate to 90% saturation with stirring. The precipitate was separated off by centrifugation and was redissolved in the standard buffer. It was then desalted.

Gel filtration was conducted on a 2. 6 × 70 cm column of Sephadex G-50 in the standard buffer at a rate of flow of 55 ml/h. The fraction volume was 9.2 ml. The amounts of protein in the fractions were determined by Bradford's method, with the staining of the protein in a 0.05 M solution of Coomassie G-250 in 3.5% $HClO_4$.

Synthesis of the Affinity Sorbent. Agarose A-6 (100 ml) was washed with a fourfold volume of H_2O. The gel was placed in a 0.5-liter round-bottomed flask, and 30 ml of NaOH*, diglycidyl ether†, and 0.2 g of $NaBH_4$ were added. The reaction mixture was stirred with a rotor at 60°C for 1 h and the gel was then washed with water to pH 7. With stirring, a

*Concentration not given.

†Amount not given.

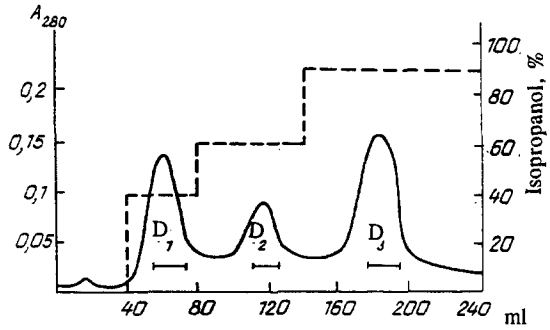

Fig. 3. Hydrophobic chromatography of fraction C_2 on a column (1.0 × 15 cm) of Polikhrom in 0;05 M Tris-HCl, pH 6.0. Rate of flow 10 ml/h.

solution of 300 mg of ABA in a mixture of 20 ml of DMFA and 10 ml of water was added to the gel at pH 13, and the mixture was incubated at room temperature for 24 h. After incubation,the sorbent was washed with 50% DMFA and then with water. The oxirane groupings that had not reacted were blocked with 2-aminoethanol.

Affinity chromatography was condcted on a 1. 5 × 10 cm column with ABA-agarose in 0.05 M Tris-HCl, 1 M NaCl, buffer at a rate flow of from 10 to 30 ml/h.

Hydrophobic chromatography was conducted on a column of the sorbent Polikhrom-1. The freeze-dried protein was dissolved in 1 ml of 0.05 M Tris-HCl buffer containing 0.2 M NaCl, pH 7.5, and was deposited on the column. Elution was performed in a gradient of isopropanol: 20-40-60-80-90%. The experiments on the binding of [^{3}H]ABA were performed by filtration through Millipor nitrocellulose filters (Germany). Micro test-tubes were each charged with 10 μl of [^{3}H]ABA to a final concentration of 10^{-6} M, and then 10 μl of water was added to the control or 10 μl of a solution of unlabeled ABA in a concentration of 10 M to determine the level of nonspecific binding. After this, 80 μl of the protein preparation in Tris buffer was added. The mixture was incubated at 20°C for 30 min, and the proteins were separated from the nonbound [^{3}H]ABA by filtration under vacuum. The filters were then dried and were placed in bottles with the toluene scintillator ZhS-106, and radioactivity was determiend on a Beta-1 counter.

Electrophoresis was conducted by Laemmli's method under denaturing conditions with a linear increase in the concentration of the PAAG gel of from 9 to 25%. The molecular mass was determined from the results obtained after electrophoresis, with the construction of a curve using the method of least squares. The following were used as markers: 1) a cyanogen bromide hydrolysate of myoglobin with mol. masses of 17, 14, 8, 6, and 2.5 kDa; 2) BSA, 68 kDa; and 3) ovalbumin, 43 kDa.

REFERENCES

1. A. I. Markova, A. K. Tonkikh, and Sh. I. Salikhov, Fiziol. Rast., **37**, No. 4, 103 (1990).
2. Kh. K. Avlyanov, O. N. Veshkurova, A. A. Takanaev, and Sh. I. Salikhov, Khim. Prir. Soedin., No. 6, 702 (1992).
3. Ya. S. Ziyaev, O. N. Veshkurova, A. A. Takanaev, and Sh. I. Salikhov, Khim. Prir. Soedin., No. 1, 92 (1994) [in this issue].
4. M. Kh. Abidova, G. T. Mavlyanov, and I. I. Grigina, Dokl. Akad. Nauk UzSSR, No. 11, 52 (1990).
5. Kh. K. Avlyanov, O. N. Veshkurova, A. A. Takanaev, and Sh. I. Salikhov, Khim. Prir. Soedin., No. 6, 699 (1992).