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AFFINITY SORBENT FROM ABSCISIC ACID — AGAROSE A-6

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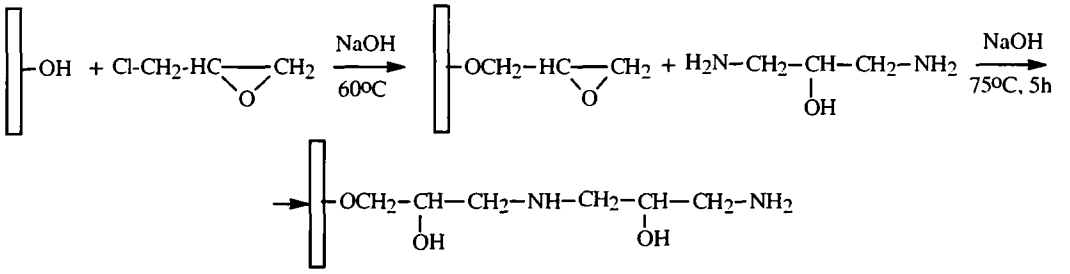
Optimal conditions are found for synthesizing an affinity sorbent from abscisic acid (ABA) and aminopropylagarose A-6. A protein with high specific affinity for ABA is isolated from the microsomal fraction of four-day cotton sprouts using the synthesized sorbent.

Key words: affinity sorbent, immobilized ^{3}H-ABA, abscisic acid—aminopropylagarose A-6.

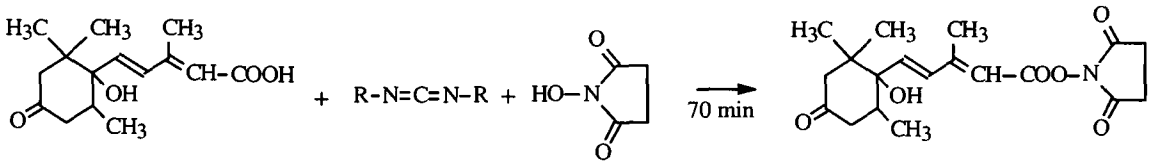
The isolation of hormone-binding proteins from plant material is known to be exceedingly labor-intensive [1-6]. The purification of these proteins includes both traditional methods of liquid chromatography (gel, ion-exchange, distribution, and adsorption) and affinity chromatography. The use of affinity sorbents with immobilized phytohormones enables one or another hormone-binding protein to be isolated and easily purified.

One of the most important phytohormones that is involved in growth, development, and aging of plants is abscisic acid (ABA) [2, 3]. In order to isolate the cotton proteins that are responsible for binding this phytohormone, we synthesized an affinity sorbent by immobilizing ABA on agarose A-6 using the following scheme:

1. Addition of spacer to matrix



2. Preparation of succinimide derivative of ABA



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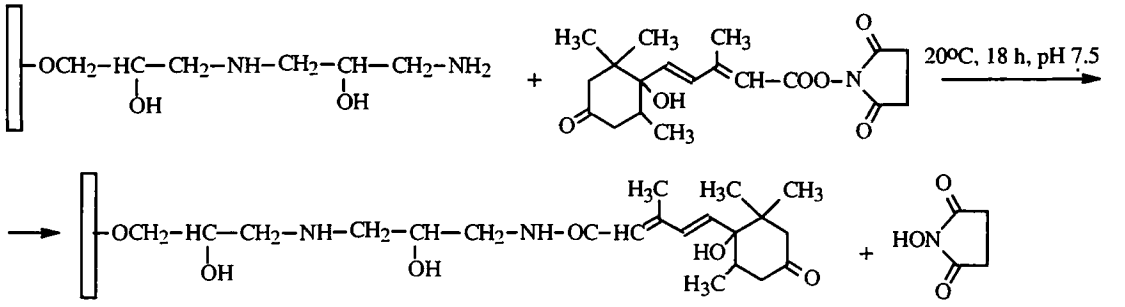
3. Addition of ligand to matrix



The amount of ^{3}H-ABA bound to aminopropylagarose A-6 was determined by β-counting; the capacity of synthesized sorbents, by column chromatography; the amount of protein in the eluent, by the Lowry method. The data are presented in Table 1.

Table 1 shows that the amount of bound ^{3}H-ABA increases with increasing amount of added ^{3}H-ABA in all experiments. The capacity for protein of the synthesized sorbents increases only until the third experiment. It remains constant in the fourth and fifth. This is explained by steric hindrance toward formation of the phytohormone complex with the specific protein.

Thus, the optimal conditions for immobilizing ^{3}H-ABA on aminopropylagarose A-6 include the addition of ^{3}H-ABA (2 mg) to a suspension of the sorbent (1 ml). In this instance, ^{3}H-ABA (1.0 mg) is bound and the capacity for protein is 12.50 μg per 1 ml of affinity sorbent.

These optimal conditions were used for preparative synthesis of ABA—aminopropylagarose A-6. A column packed with the synthesized sorbent was used to isolate ABA-binding proteins from four-day cotton sprouts.

The quality of the sorbents was checked and the selectivity was determined by HPLC analysis of the isolated ABA-binding proteins (Fig. 1). This produced three protein fractions, which were checked for ability to bind specifically to ^{3}H-ABA on "Schleicher and Schull" nitrocellulose filters. Only the second fraction had the ability to bind. According to HPLC analysis, this fraction contained 90% of the total amount of desorbed protein.

Thus, the affinity sorbent synthesized by us, ABA-aminopropylagarose A-6, is suitable for isolating ABA-binding cotton proteins and apparently can be used to isolate ABA-binding proteins from other plant material.

EXPERIMENTAL

Activation of Agarose A-6 with Epichlorohydrin. Agarose A-6 (50 ml) was washed in a Schott funnel, transfered to a cylinder, suspended in distilled water (70 ml), and treated with NaOH (10 ml, 0.6 M) and epichlorohydrin (5 ml). The mixture was left for 30 min at room temperature. The cylinder was heated to 60°C on a water bath for 2 h with constant stirring. The solid was washed with distilled water until the pH was 7.

Addition of Spacer. Epoxy-activated agarose A-6 (50 ml) was equilibrated with NaOH (0.1 M) and treated with 1,3-diaminopropan-2-ol (0.1 g). The reaction proceeded for 5 h at 75°C. After incubation the sorbent was washed to remove unbound 1,3-diaminopropan-2-ol with NaCl solution (1 M) and distilled water until the pH was 7.

Preparation of Succinimide Derivative of ABA. ABA (1.4 g) and N-hydroxysuccinimide (1.35 g) were dissolved in dioxane (60 ml). The solution was treated with N,N-dicyclohexylcarbodiimide (1.85 g). After 70 min the precipitate of dicyclohexylurea was filtered off. The filtrate, which contained the N-hydroxysuccinimide derivative of ABA, was used in the next step.

Addition of Ligand. The filtrate was added to aminopropylagarose (50 ml) in phosphate buffer (0.1 M, pH 7.5) and reacted for 18 h at room temperature. The sorbent was washed with NaCl solution (1 M) and distilled water.

Selection of Optimal Conditions. The optimal immobilization conditions were selected by adding filtrate (0.043, 0.064, 0.086, 0.129, and 0.172 ml) with ^{3}H-ABA (23.2 mg/ml, determined by radioactivity) from the second synthesis step. After the reaction was finished, the sorbents were washed until no radioactivity was detected in the washings.

TABLE 1. Dependence of Capacity of Synthesized Affinity Sorbent ^{3}H-ABA—Aminopropylagarose A-6 (1 ml) on Amount of Added and Bound ^{3}H-ABA

Expt. No.	^{3}H-ABA					ABA-binding protein, μg
	added			bound		
	mg	ml	counts/min	mg	counts/min	
1	1	0.043	1.1×10^8	0.5	0.55×10^8	6.25
2	1.5	0.064	1.65×10^8	0.8	0.88×10^8	7.8
3	2	0.086	2.2×10^8	1.0	1.1×10^8	12.5
4	3	0.129	3.3×10^8	1.5	1.65×10^8	12.5
5	4	0.172	4.4×10^8	2.4	2.64×10^8	12.5

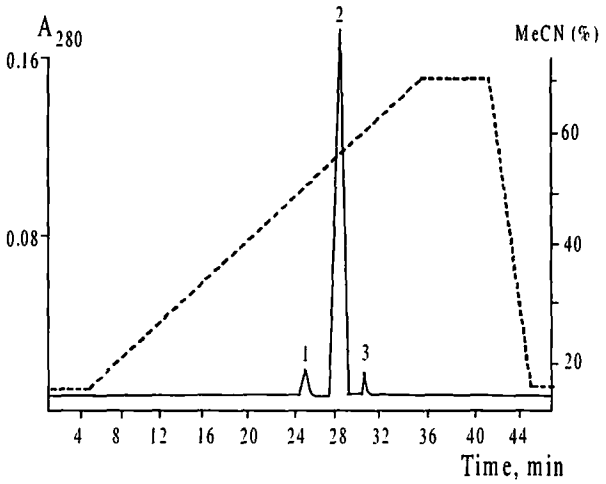


Fig. 1. HPLC analysis of isolated proteins on ^{3}H-ABA—aminopropylagarose A-6. For analytical conditions, see Experimental.

Determination of Amount of Bound ^{3}H-ABA. A suspension of ^{3}H-ABA—aminohexylagarose A-6 (1 ml) was dried in air and quantitatively transfered into scintillation cocktail ZhS-1. The radioactivity was measured in a β-counter. Aminohexylagarose A-6 through which the corresponding amount of ^{3}H-ABA had been passed without carrying out the reaction and which had been washed as in the experiments was used as the control. The difference in the amounts of radioactivity in the control and samples was used to calculate the amount of bound ^{3}H-ABA using the formula

$$C = (A_1 - A_2) + C_0/B_0,$$

where C is the concentration of bound ^{3}H-ABA, C_0 is the concentration of added ^{3}H-ABA, A_1 and A_2 are the counts per minute in the samples and control, and B_0 is the counts per minute of ^{3}H-ABA added to the reaction.

Determination of Capacity of Synthesized Sorbent. The microsomal fraction from subcellular fractionation of homogenized four-day cotton sprouts was solubilized using triton X-100 and irradiated with ultrasound. Components less than 1 and more than 300 kDa were removed by ultrafiltration using a YM2 Diaflo membrane and an Amicon XM300 filter (USA). The filtrate was placed on a column (0.5 × 3.0 cm) with ABA-aminopropylagarose A-6 (1 ml of suspension) equilibrated with tris-HCl buffer (50 mM) containing saccharose (50 mM), $MgCl_2$ (0.2 mM), EDTA (0.1 mM), and triton X-100 (0.05%) at pH 6.0. Nonbound substances were washed out with the same buffer at 10 ml/h. Bound substances were eluted by NaCl (1 M) in the same buffer.

Sorption of specific protein was determined on a DuPont 8800 chromatograph (USA) with a 250/8/4 Nucleosil 100-5 C_{18} column. Buffer A was 0.1% CF_3COOH; B, CH_3CN. The flow rate was 60 ml/h. UV detection was at 280 nm with

sensitivity 0.16 optical units. The gradient was (%B/min): 5/0-5; 60/35-40; 5/45. The resulting fractions were tested for binding with ^{3}H-ABA on "Schleicher and Schull" (Germany) nitrocellulose filters.

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