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ISOLATION AND INVESTIGATION OF THE ETHYLENE PRODUCING ENZYME OF COTTON

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The activity of the ethylene-producing enzyme (EPE) in a homogenate of cotton leaves and in fractions obtained via differential centrifugation and fractional precipitation is studied. A model system is developed for testing EPE. The effect of known activators and inhibitors of ethylene biosynthesis on the EPE activity is studied. Chromatographic methods are used to prepare the highly purified enzyme from the water-soluble fraction of cotton proteins and to identify it as ACC-oxidase. The enzyme has MM ~38 kDa and converts aminocyclopropanecarboxylic acid (ACC) into ethylene.

Many researchers have recently turned their attention to the ethylene-hormone, which regulates practically all development stages of a plant. The ethylene-producing enzyme (EPE) ACC-oxidase (ACC = 1-aminocyclopropanecarboxylic acid) plays a fundamental role in the biosynthesis of ethylene. It catalyzes the complicated reaction of producing ethylene from ACC [1].

A homogeneous form of EPE has been isolated from apple and cantaloupe tissues [2]. However, the structure and function of EPE from cotton is also interesting because ethylene initiates such an important physiological process as the shedding of leaves [3]. It is supposed that EPE is an oxidase and that several cofactors are necessary for its activity, like for other known oxidases [4].

Cotton leaves were homogenized in 0.1 phosphate buffer (pH 7.0) in order to isolate the enzyme. The resulting homogeneous mixture did not produce ethylene. However, ethylene was released (13 nl/g) when substrate (5 μM ACC) was added. The mixture was separated by differential centrifugation to remove cell walls, nuclei, and other organelles. Occurrence of the enzyme in the resulting fractions was studied.

The EPE was found in the supernatant, which contained the endoplasmic reticulum and soluble proteins, after centrifugation at 12,000 g. The protein fraction that was precipitated by ammonium sulfate was not active toward ethylene production even in the presence of ACC. This may be due to the fact that low-molecular-weight compounds, cofactors necessary for the ethylene-producing activity to appear, remained in the solution.

In order to answer this question, we investigated the effect on ethylene production of various cofactors that are known from the literature to participate in ethylene biosynthesis (Table 1). The results show that the basic reaction medium (BRM) that is optimal for EPE activity contains 10 mM ACC, 0.4 mM pyridoxal phosphate, 0.2 mM indolyl-3-acetic acid (IAA), 0.3 mM 2,4-dichlorophenol, and 0.3 mM iron chloride.

Ethylene production sharply decreased in the absence of pyridoxal phosphate, which is a cofactor of all transaminases. These enzymes catalyze the loss of amino groups, in this instance from ACC. Ethylene is released into the reaction medium without added ACC evidently owing to endogeneous ACC. The most important cofactors are 2,4-dichlorophenol and IAA, without which ethylene is not produced. This indicates that the desired enzyme is an oxidase. Adding iron to the reaction medium also enhances ethylene release because divalent iron is a cofactor of ACC-oxidase isolated from other plant sources [1]. Thus, it can be concluded that EPE occurs in the cytosol from plant cells, i.e., EPE is localized in the endoplasmic reticulum in addition to membranes. The results enabled a procedure for isolating and purifying EPE from cotton leaves to be developed.

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TABLE 1. Effect of Activators and Inhibitors of Ethylene Biosynthesis on EPE Activity

Reaction mixture	Ethylene production (%) per time interval, h		
	1	3	24
Basic reaction medium (BRM)	100.00	100.00	100.00
BRM without pyridoxal phosphate	32.05	47.39	56.63
BRM without ACC	28.21	88.30	90.00
BRM without protein fraction	32.05	65.07	82.55
BRM without IAA	33.69	33.02	38.58
BRM without Fe ions	66.86	77.45	91.13
BRM without 2,4-dichlorophenol	0	0	0

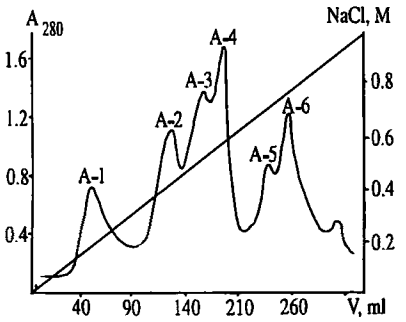

Fig. 1

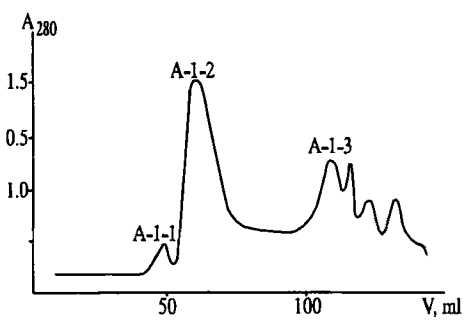

Fig.2

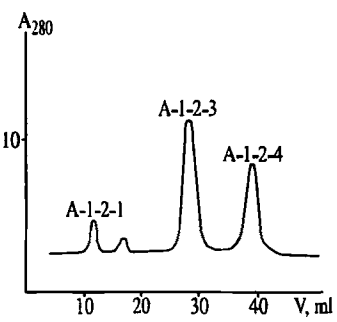

Fig.3

Fig. 1. Ion-exchange chromatography on a DEAE-cellulose column of water-soluble proteins from cotton leaves.
Fig. 2. Gel-filtration on a TSK-gel HW-50 column of fraction A-1.
Fig. 3. Gel-chromatography on a Sephadex G-150 column of fraction A-1-2.

Cotton leaves were homogenized in 0.1 M phosphate buffer. The water-soluble proteins were isolated by precipitation with ammonium sulfate (80% saturated). This fraction after desalting was chromatographed on DEAE-cellulose using a NaCl gradient (Fig. 1). Eight fractions were obtained. Of these, only the first, A-1, exhibited enzyme activity. Fraction A-1 was eluted by 0.15-0.25 M NaCl. Electrophoresis on polyacrylamide gel revealed that six proteins with MM from 15 to 60 kDa were present in this fraction. Therefore, this fraction was then chromatographed on TSK-gel HW-50 (Fig. 2). Only fraction A-1-2 of the six protein fractions was active toward ethylene production. According to electrophoretic separation on polyacrylamide gel, fraction A-1-2 consisted of five proteins that differed in quantity and MM. Then this fraction was also purified by gel-filtration on Sephadex G-150 (Fig. 3).

Only fraction A-1-2-3, the third of four fractions, exhibited ethylene-producing activity. Electrophoresis on polyacrylamide gel showed that this fraction is a homogeneous protein with MM 39 kDa and an activity of 11.3 μl C_2H_4 per mg of protein. Results of the EPE isolation from cotton leaves are given below:

Isolation step	Activity, nmol/min/mg
Homogenate after centrifugation at 18,000 rpm	0.7
Precip. after $(NH_4)_2SO_4$ treatment	1.17
Fraction 1 after ion-exchange chromatogr. on DEAE-cellulose	2.19
Fraction 2 after chromatogr. on TSK-gel	7.8
Fraction 3 after chromatogr. on Sephadex G-150	8.3

Only dansyl derivatives of ε-lysine and *o*-tyrosine were observed by analysis of the N-terminal aminoacid. Thus,

chromatographic methods were used to isolate in a homogeneous state from the water-soluble fraction of cotton proteins the enzyme responsible for conversion of ACC to ethylene. An understanding of the nature of the ethylene-producing enzyme would enable chemical preparations effecting defoliation to be used more rationally. Molecular probes for creating transgenic varieties with controlled shedding of leaves could be developed on the basis of the isolated homogeneous enzyme.

EXPERIMENTAL

Isolation of Water-Soluble Protein Fraction from Cotton Leaves. Cotton leaves (Andizhan-6 variety) in phase 2-3 of boll opening were washed with distilled water, dried, and weighed. They were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.0) at 8,000 rpm. Phenolic substances in the homogenate were adsorbed by adding nylon powder calculated to be 30% of the batch weight. The homogenization and all subsequent purification steps were performed at 4°C.

The homogenate was filtered through a double layer of cloth and centrifuged at 18,000 rpm for 30 min. The precipitate was discarded. Proteins in the supernatant were concentrated by ammonium sulfate (80% saturated) salting out. A precipitate formed overnight. It was separated from the supernatant by centrifugation at 6,000 rpm for 30 min. The precipitate was dissolved in water and dialyzed against water for 1 d, then against 50 mM Tris-HCl buffer (pH 7.5).

Ion-exchange chromatography was performed on an activated DEAE-cellulose column (2.5×8 cm) using a NaCl gradient from 0 to 1 M in 50 mM Tris-HCl buffer (pH 7.5). The flow rate was 19.2 ml/h. Volumes of the fractions were 4.8 ml.

Gel-filtration of fraction A-1-2, obtained after separation of proteins on DEAE-cellulose, was performed on a TSK-gel HW-50 column (1.5×84 cm). The proteins were eluted with 20 mM phosphate buffer (pH 7.0) at 10.2 ml/h. Volumes of fractions were 3.4 ml.

Gel-chromatography of fraction A-1-2-3 was carried out on a Sephadex G-150 column (1.0×50 cm) in 0.05 M sodium acetate (pH 7.0). The elution rate was 16.0 ml/h. Volumes of fractions were 4.8 ml.

EPE activity was determined using a basic reaction medium of the following composition: 10 mM ACC, 0.4 mM pyridoxal phosphate. 0.2 mM IAA, 0.3 mM 2,4-dichlorophenol, and 0.3 mM manganese chloride. The volume of the reaction medium was 2 ml. The reaction was carried out in the dark at room temperature. The amount of ethylene released was determined by gas chromatography 24 h after the start of the reaction on a Khrom-5 GLC (3 m column, Porapak K packing, 40-50°C) [5].

Protein was determined by the Lawry method using BSA as the standard [6].

Electrophoresis was performed by the Laemmli method in a PAAG gradient (from 9 to 25%) with added sodium dodecylsulfate [7].

N-Terminal aminoacids were determined by the Gray method [8].

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