Chemistry of Natural Compounds, Vol. 38, No. 5, 2002

IDENTIFICATION OF IAA-OXIDASE IN PEROXIDASE ISOFORMS FROM COTTON LEAVES

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UDC 577.15.158

IAA-oxidase from peroxidase of cotton leaves was identified and isolated. It was shown that IAA-oxidase is bound with ^{3}H-IAA with K_d 2×10^{-6} M. The binding is reversible, saturated, and inhibited by naphthylacetic acid and benzidine.

Key words: IAA, IAA-oxidase, peroxidase, cotton.

Plant peroxidases catalyze the oxidation of indole-acetic acid (IAA) by molecular oxygen [1]. Peroxidases from other sources, cytochrome C peroxidase and fungal and microbial peroxidases, do not oxidize IAA in the absence of H_2O_2. It has been found that two paths of IAA oxidation exist. These are metabolic with involvement of H_2O_2 and with involvement of oxygen [2]. Evidently one of the functions of plant peroxidase is to regulate the IAA hormonal level by oxidizing it to inactive 3-methyleneoxyindole.

An analysis of the amino-acid sequence of IAA-binding proteins and plant peroxidase using computing methods revealed five structurally similar fragments that are absent in peroxidase from other sources [3, 4].

We performed frontal electrophoresis in alkaline (pH 8.9) 7.5% polyacrylamide gel with subsequent identification of cotton-peroxidase auxin-binding isoforms using ^{3}H-IAA in order to provide evidence for their existence. We investigated two enzyme preparations from cotton leaves. One of these contained the corresponding polyphenols. They were removed from the other by treatment with polyvinylpyrrolidone. The preparations were incubated for 30 min with ^{3}H-IAA and separated by electrophoresis. The isoforms were colored by a solution of the chromogenic substrate benzidine chloride. Figure 1 shows the densitogram from electrophoresis of peroxidase from leaves that contained polyphenols (a). This separated into three isoforms of different relative electrophoretic mobility (REM). Peroxidase from cotton leaves that did not contain polyphenols (b) separated into two isoforms. We determined the ^{3}H-IAA binding to the isoforms of peroxidases using liquid scintillation counting.

The results show that the isoform of peroxidase from cotton leaves that did not contain polyphenols (REM 0.41) had the highest auxin-binding capability. Evidently the polyphenols compete with ^{3}H-IAA for binding sites in the enzyme preparation because it was found previously that natural polyphenols are IAA-oxidase inhibitors [5].

Enzyme	Relative electrophoretic mobility of isoforms	Radioactivity, imp./min
Peroxidase from leaves, containing	0.41	2773±139
polyphenols	0.45	2694±135
	0.52	2511±126
Peroxidase from leaves, not containing		
polyphenols	0.41	5007±250
	0.45	2661±133
Radioactive background of gel		2300±115

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 0009-3130/02/3805-0458$27.00

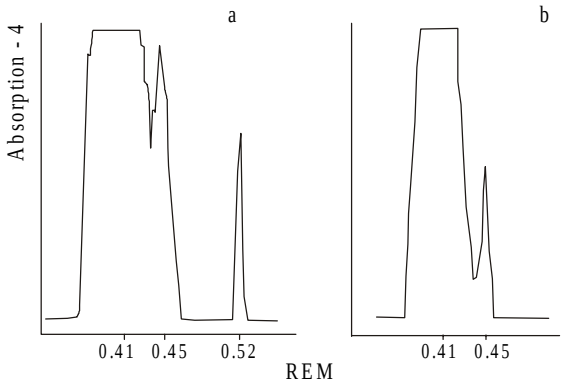

Fig. 1. Densitogram of electrophoresis in 7.5% PAAG (pH 8.9). Peroxidase from cotton leaves containing (a) and not containing (b) polyphenols.

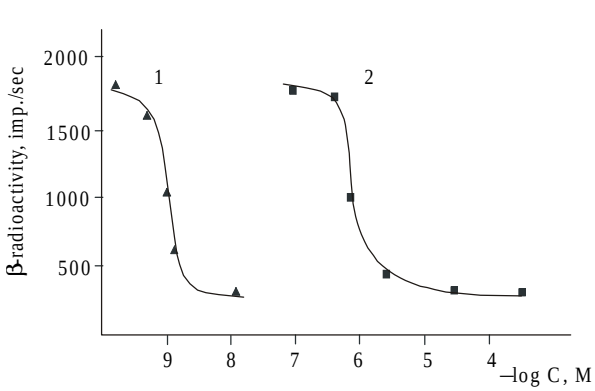

Fig. 2

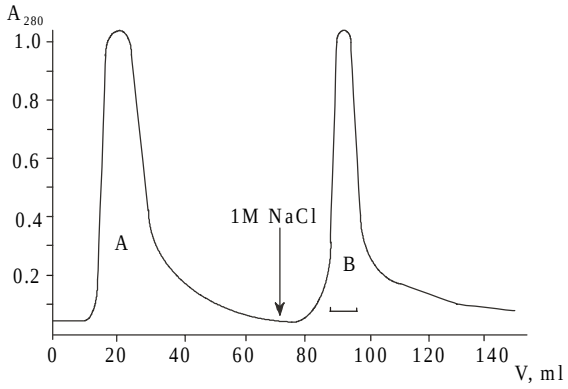

Fig. 3

Fig. 2. Specific binding of ^{3}H-IAA (30 nM) in the presence of various concentrations of unlabeled IAA. With the fraction of IAA-binding protein from cotton sprouts (1) and with peroxidase preparation from cotton leaves (2).

Fig. 3. Isolation of IAA-oxidase by affinity chromatography of peroxidase from cotton leaves not containing polyphenols over a column (10×2.6 cm) with IAA-aminohexylagarose. Protein fraction not binding with IAA-aminohexylagarose in tris-HCl buffer (0.005 M, pH 7.5) (A) and fraction of IAA-oxidase eluted by NaCl (1 M) (B).

Next, binding constants of ^{3}H-IAA to peroxidase and the cotton auxin receptor that was isolated by a previously developed method [6] were determined comparatively. The binding was performed in the presence of increasing concentrations of unlabeled IAA (from 10^{-9} to 10^{-3} M).

Figure 2 shows that ^{3}H-IAA is bound specifically with saturation to peroxidase from cotton leaves. However, the binding strength is three orders of magnitude less than to the auxin receptor (K_d 2×10^{-6} and 6×10^{-9} M, respectively).

Adding a 1000-fold excess of the specific substrates benzidine or α-naphthylacetic acid (NAA) to the analyzed sample inhibits the enzyme and partially inhibits ^{3}H-IAA binding. It should be noted that NAA, a structural analog of IAA, competes weakly in experiments on ^{3}H-IAA binding. This correlates with its effect on peroxidase in physiological tests [7].

The results indicate that peroxidase is not a true auxin receptor. In fact, according to the literature [8], not only phytohormone receptors but also transport and storage proteins in addition to biosynthesis and phytohormone-metabolism proteins possess hormone-binding capability. Auxin binding by the recognition segment of the enzyme is apparently necessary for the correct orientation of the molecule during its oxidation at the catalytic center.

A biospecific sorbent was synthesized to isolate IAA-oxidase by conjugating IAA to AH-agarose [9]. Peroxidase from cotton leaves was placed on a column with IAA-aminohexylagarose. The column was eluted until an eluate with a constant optical density at 280 nm was obtained. A protein fraction that did not bind to the affinity sorbent eluted (peak A). The binding

proteins (peak B) were eluted by NaCl (1 M) (Fig. 3). The resulting eluate was desalted by dialysis and lyophilized. The protein content and peroxidase activity were determined. Data for the peroxidase activity of the enzyme preparation of cotton-leaf peroxidase before placing it on the column with IAA-aminohexylagarose and after elution from it by NaCl (1 M) were obtained.

Enzyme-preparation fraction	Specific peroxidase activity, ncat/mg	Protein amount, mg
Before placement	0.021	700
Not binding to IAA-aminohexylagarose	0.042	546
Binding to IAA-aminohexylagarose	0.095	135

It can be seen that IAA-oxidase makes up about 20% of the total peroxidase. The peroxidase activity of protein eluted from the IAA-aminohexylagarose increased 4.5 times compared with its initial activity.

Thus, several features, e.g., binding to ^{3}H-IAA in vitro, binding to IAA-aminohexylagarose, and frontal electrophoresis, lead to the conclusion that the studied isoform of peroxidase is an IAA-oxidase.

EXPERIMENTAL

Leaves from the lower levels of a mature cotton plant (*Gossypium hirsutum* L., variety 175-F) were used.

Peroxidase Isolation. Leaves were ground in cold tris-HCl buffer (0.1 M, pH 6.0, 1:4 ratio) after preliminary disruption of cells by liquid nitrogen. Polyphenols were removed from the solution by treatment with DEAE-sephadex for 1 h and centrifugation at 7000 rpm for 20 min. The supernatant was treated with ammonium sulfate (80% saturated) and left at 4°C for 14 h. The resulting precipitate was chromatographed over a column packed with Toyopearl HW 55F (1.5×150 cm) equilibrated with tris-HCl (0.1 M, pH 6.0). The separation was performed in this same buffer. Fractions (5 mL) were collected. The enzyme activity in each was analyzed. The protein concentration was determined by the Lowry method [10]; peroxidase activity, by the literature method [11].

Electrophoretic separation of peroxidase was performed in alkaline (pH 8.9) 7.5% polyacrylamide gel by a previously described method [12]. The gel was developed by benzidine solution (1%).

Determination of ^{3}H-IAA Binding by Isoforms. Labeled IAA (30 nM) was added to the peroxidase preparations (30-50 μg of protein). The mixture was incubated for 30 min and then examined by electrophoresis [12]. Isoforms were developed by benzidine solution (1%). Colored bands of the isoforms were cut out (0.5 cm by 1 cm), placed into vials, and treated with H_2O_2:NH_4OH (95:1, 0.5 mL). They were stored for 1-3 h at 0°C and then 10 h at 50°C. The dissolved gel bands were added to ZhS-106 scintillation cocktail. The radioactivity was counted on a Beta-1 counter.

The specific binding of peroxidase from cotton leaves to ^{3}H-IAA was studied using filtration on Synpor (Czech Rep.) nitrocellulose filters (24 mm diameter, pore size 0.4-0.6 μm) as described before [13]. The binding curve was plotted using a single concentration (30 nM) of labeled IAA and various concentrations of a 1000-fold excess of unlabeled IAA.

IAA-oxidase was isolated by affinity chromatography over a column packed with IAA-aminohexylagarose (1.5×10 cm). Peroxidase from cotton leaves was dissolved in buffer (tris-HCl, 50 mM, pH 7.4) and placed on the column. Nonbinding proteins were eluted by the same buffer; binding ones, by NaCl (1 M). Both were dialyzed and lyophilized.

ACKNOWLEDGMENT

The work was performed with financial support from the STCU, Grant Uzb-75.

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