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# IDENTIFICATION OF COTTON PR-PROTEINS EXHIBITING 1,3-β-GLUCANASE AND CHITINASE ACTIVITY

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*The biosynthesis and spectrum of PR-proteins isolated from leaves of eight cotton varieties that were affected by* Verticillium dahliae *were studied. It was shown that 1,3-β-glucanase, chitinase, and catalase activity increased in response to the pathogen. 1,3-β-Glucanase, three of its isoforms, and chitinase were isolated.*

**Key words:** cotton, pathogen, PR-proteins, enzymes, chitinase, 1,3-β-glucanase, catalase.

Plant stress proteins have recently been widely studied. PR (pathogensis-related) proteins, the formation of which is induced by many pathogenic and apathogenic factors, are some of the more important ones [1-3]. Stress proteins were previously considered to be devoid of enzymatic activity. However, peroxidase, chitinase, and 1,3-β-glucanase activity has recently been observed in these proteins [4-6]. The level of PR-protein biosynthesis and resistance of plants to stress factors are definitely related. This makes stress proteins useful as markers of plant resistance to biotic and abiotic factors.

We selected eight cotton varieties for a study of the biosynthesis level and spectrum of PR proteins formed in response to *V. dahliae*. These were C 2609, C 4610, C 6524, Armugon, Ok dare, Namangan 77, Andizhan 9, and Andizhan 40, which differ in resistance to attack by *V. dahliae*. Table 1 gives the results for specific activity of enzymes isolated from plants attacked by *V. dahliae* 6, 12, and 20 days after pathogen innoculation.

The activity of all studied enzymes increased in response to the pathogen. The enzyme activity increased less in varieties C 4610 and Andizhan 9, which showed signs of wilt disease, i.e., in sensitive varieties, than in more resistant plants.

Molecular weights (MW) of the cotton-leaf PR proteins synthesized after *V. dahliae* attack were estimated by isolation and gel chromatography over Ultragel AcA54 in the presence of marker proteins insulin (6 kDa), cytochrome C (12.5), ovalbumin (43), and dextran blue (Fig. 1). Biosynthesis of proteins with MW < 5 kDa was inhibited upon attack of cotton by wilt. A new protein with MW > 7 kDa was synthesized. Furthermore, the amount of protein with MW 12.5 kDa increased compared with the control.

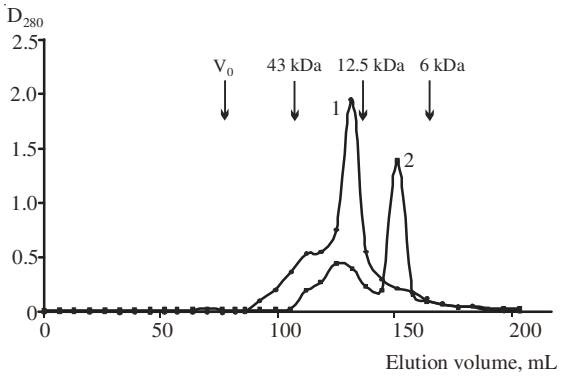


Fig. 1. Gel chromatography of cotton-leaf proteins over Ultragel AcA34: cotton PR-proteins (1), cotton-leaf proteins (2).

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TABLE 1. Glucanase, Chitinase, and Catalase Activity of Cotton-Leaf Proteins

| Cotton variety | Enzyme activity after innoculation of *V. dahliae* conidiae | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1,3-$\beta$-glucanase, U/mg protein | | | chitinase, U/mg protein | | | catalase, mcat/L | | |
| | 6 d | 12 d | 20 d | 6 d | 12 d | 20 d | 6 d | 12 d | 20 d |
| C 2609 | 0.073 | 0.111 | 0.303 | 2.07 | 7.1 | 3.15 | 39.96 | 46.62 | 213.12 |
| C4610 | 0.31 | 0.35 | 0.597 | 1.75 | 3.85 | 7.35 | 59.94 | 33.3 | 79.92 |
| C6524 | 0.23 | 0.119 | 1.3 | 3.5 | 4.9 | 6.3 | 93.24 | 13.32 | 53.28 |
| Armugon | 0.48 | 0.21 | 0.31 | 0.48 | 5.25 | 3.85 | 26.64 | 13.32 | 319.68 |
| Ok dare | 0.096 | 0.306 | 0.251 | 1.96 | 2.45 | 13.5 | 26.64 | 26.64 | 159.84 |
| Namangan 77 | 0.23 | 0.085 | 0.327 | 7.7 | 6.3 | 8.75 | 39.96 | 66.6 | 53.28 |
| Andizhan 9 | 0.33 | 0.061 | 0.63 | 8.75 | 5.25 | 7.7 | 39.96 | 66.6 | 412.92 |
| Andizhan 40 | 1.4 | 0.054 | 0.60 | 3.85 | 7.0 | 8.75 | 33.16 | 33.3 | 306.36 |

TABLE 2. Glucanase Activity of Fractions from Cotton Leaves

| Fraction | Protein concentration, μg/mL | Glucanase activity, U/mg protein |
|---|---|---|
| Cotton extract | 1600.00 | 0.023 |
| Precipitate after $(NH_4)_2SO_4$ (30% saturation) | 123.50 | - |
| Precipitate after $(NH_4)_2SO_4$ (60% saturation) | 120.00 | 0.068 |
| Fraction not sorbed on DEAE cellulose | 82.34 | 0.345 |
| Fraction I, eluted from CM-cellulose | 15.84 | 8.490 |
| Fraction II, eluted from CM-cellulose | 9.50 | 1.540 |
| Fractions obtained from HPLC chromatography | | |
| 1 | 3.20 | 6.250 |
| 2 | 9.50 | - |
| 3 | 3.20 | 7.030 |
| 4 | - | - |
| 5 | 3.20 | - |
| 6 | 15.80 | - |
| 7 | 12.66 | 0.920 |
| Fraction eluted from affinity column | 3.10 | 8.400 |

1,3-$\beta$-Glucanase was isolated by homogenizing cotton leaves 3 d after pathogen innoculation and extracting with ammonium-acetate buffer for 1 h with stirring. Proteins in the supernatant after centrifugation were precipited stepwise by ammonium sulfate. The resulting proteins were dialyzed against buffer. Total proteins were separated over a column of DEAE-cellulose using a NaCl gradient. Glucanase activity was observed in the fraction that was not sorbed on DEAE-cellulose and was further separated over a column of CM-TSK gel. Protein fractions were eluted by a NaCl gradient and analyzed by PAAG gel-electrophoresis (Fig. 2, tracks 3 and 4 in the electrophoregram). Glucanase activity was determined at each stage and was observed in the first two fractions eluted from CM-TSK gel that contained proteins with MW 30 kDa, characteristic for glucanases isolated from other plant sources. Then, these protein fractions exhibiting 1,3-$\beta$-glucanase activity were separated by HPLC (producing 7 fractions with different retention times) (Table 2).

Table 2 shows that three fractions isolated by HPLC exhibited glucanase activity. It can be assumed that the obtained fractions with different retention times contained three different glucanase isoforms. Thus, enzymes purified up to 300 times were isolated from cotton leaves innoculated with *V. dahliae*.

Affinity chromatography with immobilized laminarin as the ligand was used to obtain highly purified 1,3-$\beta$-glucanase. Proteins that were precipitated by ammonium sulfate (up to 60% saturation) were dissolved in water, dialyzed against Tris-HCl (0.05 M, pH 8.5), placed on an affinity sorbent synthesized by us in the same buffer, and eluted by NaCl solution (1 M). One fraction with high glucanase activity was obtained but in low yield (Table 2). The degree of purification was 350 times.

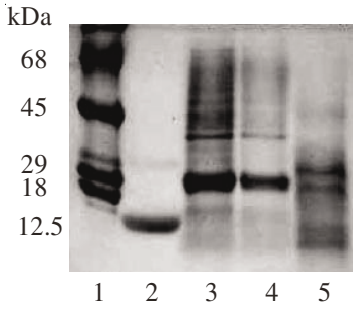


Fig. 2. Electrophoresis in PAAG (10-15%) and Na-DDS (0.1%) gradient of cotton-leaf fractions: marker proteins (lactalbumin, 18 kDa; carboanhydrase, 29; ovalbumin, 45; BSA, 67) (1), cytochrome C (2), fraction I from CM-TSK ion-exchange chromatography (3), fraction II from CM-TSK (4), fraction III from CM-TSK (5).

Chitinase was isolated from cotton leaves after attack by *V. dahliae* conidiae by two extractions in the cold by NaOAc buffer (0.1 M, pH 5). The supernatant after centrifugation was dialyzed against water. Chitinase was chromatographed over regenerated chitin prepared by the literature method [5]. Nonspecifically bound proteins were desorbed from the column using Tris-HCl solution (0.02 M, pH 7.5). Chitinase was eluted by a NaCl gradient.

Chitinase activity was determined after desalting and concentrating. Fraction 2, which exhibited chitinase activity, was treated successively with acetone (10%, 2°C), alcohol (50%, −10°C), and ammonium sulfate (up to 100% saturation, 18°C). Precipitated proteins were separated by centrifugation, dissolved in water, dialyzed, and dried. Chitinase activity of the isolated fraction was 3.5 U/mL; degree of purification, 70 times.

The observed dynamics of increased glucanase and chitinase activity in response to the pathogen led to the conclusion that these enzymes can act as markers of resistance to wilt for various cotton varieties and lines. For catalase, the resistance of cotton to wilt was not related to enzyme activity. The isolated highly purified enzymes can be used as antigens for developing immunoenzyme analyses to detect these markers during selection of new cotton varieties.

## EXPERIMENTAL

**Plant Material.** Cotton was grown in pots containing sterile soil. Plants (one-month old) were innoculated with *V. dahliae* conidiae ($2 \times 10^7$ spores/mL) in three internodal locations. Leaves (30 g each) were collected after 6, 12, and 20 d. Stress proteins were extracted from leaves by acetate buffer (0.1 M, pH 5.0). Salts and carbohydrates were removed by dialysis. Glucanase, chitinase, and catalase activity were determined.

**Gel chromatography** of cotton-leaf proteins and PR-proteins was carried out over Ultragel AcA54 (Sweden) ($1.5 \times 65$ cm) in ammonium-carbonate buffer (0.01 M, pH 8). Proteins (10 mg) in buffer (1.5 mL) were placed on the column and eluted (10.5 mL/h). Fractions (3.5 mL) were collected and analyzed on a spectrophotometer at 280 nm.

**Isolation of 1,3-$\beta$-Glucanase.** Cotton leaves (0.5 kg) that were innoculated with *V. dahliae* conidiae were extracted with ammonium-acetate buffer (2.5 L, 0.01 M) containing EDTA (1 mM), PMSF (5 mM), and dithiothreitol (1 mM) for 1 h with stirring. Proteins in the supernatant after centrifugation for 30 min at 6,000 rpm were precipitated stepwise by ammonium sulfate (up to 30 and 60% saturation). Enzyme precipitates were separated by centrifugation at 4°C for 20 min at 6,000 rpm. The precipitate obtained after precipitation at 60% saturation was dissolved in water, dialyzed against water and potassium-phosphate buffer (15 mM, pH 7.5), and placed on a DEAE-cellulose column ($2.5 \times 6$ cm). The active fraction was dialyzed against water, adjusted to NaOAc buffer (20 mM, pH 5.0), and separated over a column ($2.5 \times 8$ cm) of CM-TSK gel. Proteins were eluted by a NaCl gradient (0-1 M). Fractions were collected, dialyzed against water, and lyophilized.

**HPLC of proteins** was performed using an Agilent 1100 system (Agilent Inc., USA) and a Zorbax Eclipse 300 SB C18 column ($4.6 \times 250$ mm, 5 μm) with a linear gradient of $CH_3CN$ in TFA (0.1%, 5-65%) at flow rate 1 mL/min with photometric detection at 226 nm.

**Synthesis of Affinity Sorbent for Isolation of 1,3-$\beta$-Glucanase.** TSK HW-65 gel (10 g) was washed with distilled water; dried in air; and treated with 1,4-butanediol diglycidyl ether (1 mL), NaOH solution (2 mL, 0.3 M), and NaBH4 (4 mg). The reaction was carried out at room temperature with stirring for 15 h. The gel was washed with a large volume of distilled water, treated with Tris-HCl buffer (2 mL, 0.1 M, pH 10.5) and laminarin (2 mg), held at 50°C for 17 h, washed with buffer, treated with glycine solution (10 mL, 2 M), and left for 72 h with intermittent stirring. The resulting affinity sorbent was washed with water and used further to isolate 1,3-$\beta$-glucanase.

**Chitinase activity** was determined by hydrolysis of colloidal chitin as substrate for enzyme solution for 2 h at 37°C. The resulting reducing sugars were determined using dinitrosalicylic reagent [7]. Colloidal chitin (2 mL) was treated with potassium-phosphate buffer (1 mL, 0.2 M, pH 6) containing $CaCl_2$ (2 mM) and enzyme solution (1 mL). The hydrolysis was carried out at 37°C in a thermostat and stopped by boiling the solution for 5 min. The amount of *N*-acetylglucosamine in solution was detemrined using 3,5-dinitrosalicylic acid. A control solution was prepared in a similar manner but enzyme that was inactivated by boiling was added to it to compensate for the carbohydrates present in the solution.

**Glucanase activity** was determined from the amount of glucose produced by laminarin hydrolysis for 1 h at 37°C [8]. Laminarin solution (0.1 mL, 1%) was treated with enzyme solution (0.1 mL) and ammonium-acetate buffer (0.3 mL, 0.1 M, pH 5.0), stirred at constant temperature for 1 h, treated with Somody reagent (0.5 mL), placed for 40 min in a boiling-water bath, cooled, treated with Nelson reagent (0.5 mL), shaken, and adjusted to volume with water (up to 5 mL). Optical density was determined vs. a control at 610 nm.

**Catalase activity** was determined as before [9]. PR-protein extract (0.1 mL) was treated with $H_2O_2$ solution (2 mL, 0.03%). Distilled water (0.1 mL) was added to a blank solution instead of protein solution. The reaction was stopped after 10 min by adding ammonium molybdate solution (1 mL, 4%). The color intensity was measured on a SF-26 spectrophotometer at 410 nm vs. the control. Catalase activity was calculated using the formula

$$E = (A_{bl} - A_{ex}) \times V \times t \times K \text{ (mcat/L)},$$

where $E$ is the catalase activity (mcat/L); $A_{bl}$ and $A_{ex}$, the extinctions of the blank and experimental samples; $V$, the sample volume (0.1 mL); $t$, incubation time (600 s); $K$, millimolar extinction coefficient of $H_2O_2$ ($22.2 \times 10^3$ $mM^{-1} \times cm^{-1}$).

**Electrophoresis of proteins and peptides** was performed by the Laemmli method in a PAAG (10-15%) and Na-DDS (0.1%) gradient [10].

**Protein concentration** was determined by the Bradford method [11] using trypsin and ovalbumin as standards.

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