Chemistry of Natural Compounds, Vol. 44, No. 3, 2008

ROLE OF ANIONIC ISOFORMS OF PEROXIDASE DURING PHYTOPATHOGENIC INFECTION OF PLANTS FROM THE FAMILY MALVACEAE

A. A. Akhunov,[1*] Z. Golubenko,[1] N. R. Khashimova,[1] Yu. V. Beresneva,[1] N. A. Abdurashidova,[1] E. Ch. Mustakimova,[1] A. F. Bokov,[1] S. O. Vshivkov,[1] and R. Stipanovik[2]

UDC 577.124.5:633.511

An increase of the activity and the appearance of new isoforms (high- and low-molecular-weight) in the isospectrum of peroxidase were noted after the action of the phytopathogen Verticillium dahliae *on plants from the family Malvaceae. The temperature was shown to have an effect on the resistance to infection of the studied plants. Isoelectric focusing showed that anionic isoforms of peroxidase had isopoints 4.7 and 3.5.*

Key words: peroxidase, electrophoresis, isoelectric focusing, phytopathogenesis, Malvaceae, *Verticillium dahliae.*

A common response of plants to infection is the synthesis of specific proteins. Hypotheses about the synthesis of *de novo* isoforms of peroxidase in infected tissues have been confirmed experimentally [1-3]. Plants infected by pathogenic fungi accumulate large amounts of lignin [4]. The activity of peroxidase, which can participate in lignin synthesis, increased for certain host—pathogen interactions. Suberin, in the synthesis of which anionic peroxidases are also involved, is another defense against infection and pathogenesis [5, 6]. Plant phenolic metabolites that are toxic to fungi may also form at the same time that lignification of the cell wall occurs [7, 8].

Our goal was to study the activity and isospectrum of peroxidase from plants of the family Malvaceae growing in Uzbekistan and affected by the fungus *Verticillium dahliae*.

First we investigated four wild species of Malvaceae: *Hibiscus esculentus*, *Alcea rosea*, *Malva sylvestris*, and *M. meluca*.

We found that peroxidase activity of leaves increases after infection of the plants with *V. dahliae* (Table 1). The isoenzyme composition also changes (Fig. 1). In experiments carried out at different temperatures, changes in the overall enzyme activity were noted with increasing coloration of the individual isoforms. Moreover, a significant difference in the rate of activation and enzyme synthesis was observed. One day after infection, the activity in *M. sylvestris* and *M. meluca* increased by an average of 1.5-2 times relative to the control; for the other two studied species (*A. rosea* and *H. esculentus*), by 1.1-1.4 times.

The isospectrum of peroxidase from leaves of *H. esculentus* showed four isoforms with R_f values 0.31, 0.69, 0.72, and 0.75 (Fig. 1A). The increase of enzyme activity upon infection of plants in autumn (at air temperature 20-22°C) is probably due to a high-molecular-weight isoform with R_f 0.31. In summer (at 40-45°C), the activity of this isoform decreases after infection (Fig. 1A).

A temperature effect on activation of various peroxidase isoforms was also observed for *A. rosea*. The isospectrum of peroxidase from leaves of this species showed seven isoforms with R_f values 0.32, 0.42, 0.48, 0.51, 0.60, 0.70, and 0.74 (Fig. 1B). A *de novo* isoform with R_f 0.22 appeared in experimental samples. The activities of isoforms with R_f values 0.32, 0.42, and 0.48 increased. The activities of isoforms with R_f values 0.42 and 0.48 decreased with increasing air temperature.

1) A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, 100125, Tashkent, ul. Mirzo Ulugbeka, 83, Republic of Uzbekistan, fax: 262 70 63, e-mail: ali@ibchem.ccc.uz; 2) College-Station, Texas, USA. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 280-283, May-June, 2008. Original article submitted February 11, 2008.

 0009-3130/08/4403-0352

TABLE 1. Change of Activity of Peroxidase Isolated from Leaves of Malvaceae Infected with *V. dahliae*, E/mg

Plant	Air temperature 20-22°C		Air temperature 40-45°C	
	control	expt.	control	expt.
M. sylvestris	20.11±1.7	34.07±2.8	24.00±1.2	44.00±3.1
M. meluca	5.1±0.2	10.3±0.7	4.80±0.1	8.30±0.9
H. esculentus	21.70±1.4	23.90±1.4	19.5±1.1	20.10±1.3
Alsea rosa	20.3±1.4	24.2±1.6	17.20±0.9	23.63±1.5

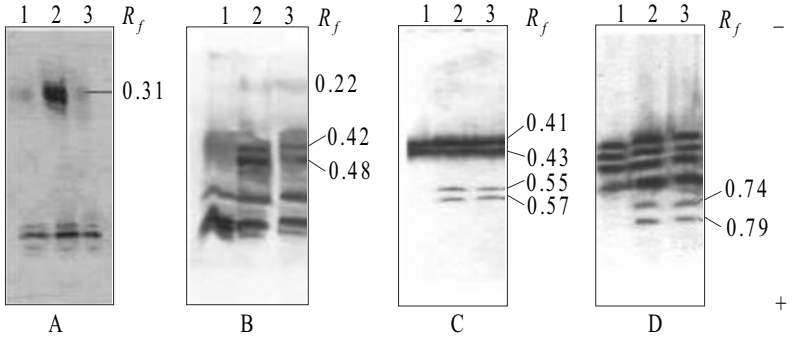

Fig. 1. Electrophoretic spectra of peroxidase from Malvaceae in PAAG (10%): *Hibiscus esculentus* (A), *Alcea rosea* (B), *Malva sylvestris* (C), *M. meluca* (D); control (1), infected with *V. dahliae* at 20-22°C (2), infected with *V. dahliae* at 40-45°C (3).

For the other two Malvaceae species, *M. sylvestris* and *M. meluca*, temperature had no affect on the peroxidase isospectrum. The peroxidase isospectrum in leaves of the *M. sylvestris* control showed two isoforms with R_f 0.41 and 0.43 (Fig. 1C). Two *de novo* isoforms with R_f 0.55 and 0.57 appeared after pathogenic infection. The control of *M. meluca* showed three isoforms with R_f values 0.50, 0.53, and 0.63. *De novo* isoforms with R_f 0.74 and 0.79 were observed in the experimental samples (Fig. 1D).

The influence of temperature on plant resistance has been reported [9-11]. According to our results, the isoperoxidase composition changed upon infection by the phytopathogen. Thus, it can be assumed that certain peroxidase isoforms play a key role in protective mechanisms of plants against pathogens. The peroxidase complex acts as a universal indicator of both biotic and abiotic stress on the plants.

Cotton is a member of the family Malvaceae and one of the most widely cultivated species in Uzbekistan. Wild cotton species are used as selection varieties to produce plants that are resistant to infection. For comparison, we investigated two wild cotton species, *Gossypium klotzschanum* and *G. raimondii*, and two industrial varieties of *G. hirsutum*, An-Bayaut-2 and C-4727. During the first day of infection, peroxidase activity in leaves of the wild cotton species increased markedly. The peroxidase activity of *G. raimondii* increased two times (control, 45.2; experimental, 88.2 E/mg); of *G. klotzschanum*, five times (control, 21.7; experimental, 113.8 E/mg). Two *de novo* isoforms with R_f 0.68 and 0.70 appeared in the isospectrum of *G. raimondii*; three isoforms with R_f 0.64, 0.66, and 0.68, for *G. klotzschanum* (Fig. 2A). At high air temperatures (40-45°C), like for *A. rosea* and *H. esculentus*, the activity and coloration of the newly appearing isoforms decreased (Fig. 1A and 1B).

Next we studied the effect of *V. dahliae* on the two cotton varieties An-Bayaut-2 and C-4727 during flowering and fruit setting. Peroxidase activity in leaves during flowering was greater than during fruit setting. In the first day after infection, the activity increased by 2.2 times for An-Bayaut-2; by 1.2 times, for C-4727 (Table 2). On the fifth day, the activity was close to that of the control for An-Bayaut-2. It continued to increase for C-4727 (twice the control value), i.e., the plant continued to battle the phytopathogen. The enzyme activity in the studied varieties increased and decreased during fruit setting, like during flowering (Table 2).

TABLE 2. Specific Activity of Peroxidase from Leaves during Different Vegetative Stages of Cotton after Infection by *V. dahliae*, E/mg

Cotton variety	Vegetative stage			Fruit-setting stage		
	control	1st day after infection	5th day	control	1st day after infection	5th day
An-Bayaut-2	46.0±3.9	105.3±8.2	56.1±4.9	22.0±1.7	35.0±2.1	18.01±0.8
C-4727	25.2±1.7	30.0±2.8	52.0±4.8	12.3±1.0	15.0±1.1	25.0±1.9

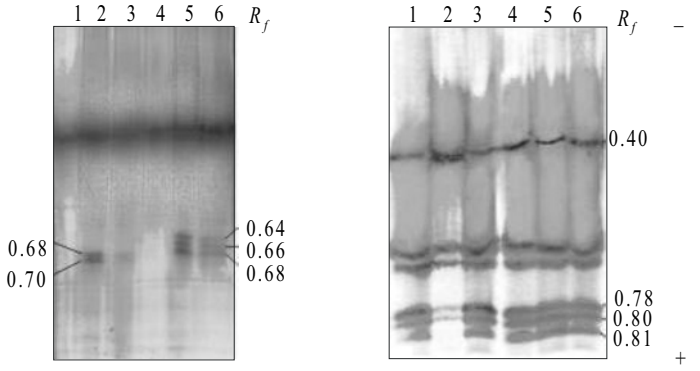

Fig. 2

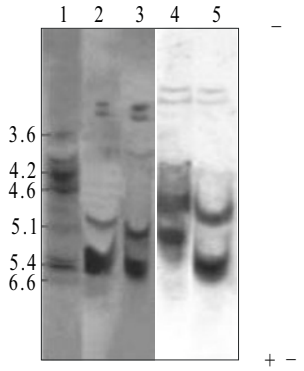

Fig. 3

Fig. 2. Electrophoretic spectra of cotton peroxidase in PAAG (10%): *G. raimondii* Ulbr (1, 2, 3), *G. klotzschianum* Andress (3, 4, 5), control (1, 4), infected with *V. dahliae* at 20-22°C (2, 5), infected with *V. dahliae* at 40-45°C (3, 6) (A); An-Bayaut-2 (1, 2, 3) and C-4727 (4, 5, 6), control (1, 4), first day after infection (2, 5), fifth day after infection (3, 6) (B).
Fig. 3. Isoelectric points of isoenzymes from total peroxidase of cotton variety An-Bayaut-2 (2, 3) and C-4727 (4, 5): IEF indicators in pH range 3.6-6.6 (1), control (2, 4), experimental (3, 5).

The enzyme system, in particular, peroxidase, must be activated to create a protective barrier between the pathogen and the host plant. Lignification, in the formation of which peroxidase participates, as noted above, is important in controlling the protective responses of the plants to infection [12] and to the development of various specific and nonspecific infections [13, 14]. Lignin deposition in resistant plants is faster than in receptive ones. According to Beckman [15], tylose formation was observed in resistant plants on day 3-4; in receptive ones, on day 10-12. The phytopathogen had distributed over the whole plant on day 10-12 whereas it was localized at the site of infection in resistant ones. The peroxidase activity change in leaves of industrial varieties An-Bayaut-2 and C-4727 leads to the conclusion that An-Bayaut-2 reacts to introduction of *V. dahliae* faster than C-4727.

Electrophoretic investigation of the peroxidase isospectrum from cotton leaves indicated that six isoforms were present in both varieties with R_f values 0.40, 0.62, 0.66, 0.78, 0.81, and 0.84 (Fig. 2B). The isoform with R_f 0.40 was activated and the activity of isoforms with R_f values 0.78, 0.81, and 0.84 decreased upon infection with the pathogen in the first day for An-Bayaut-2 according to the rate of appearance of coloration by benzidine. The activity and concentration of the isoform with R_f 0.40 was much less for C-4727 than for the control. However, isoforms with R_f values 0.78, 0.18, and 0.84 were activated (Fig. 2B).

The activated isoform with R_f 0.40 from An-Bayaut-2 was extracted from the PAAG plate and investigated by capillary electrophoresis. The electrophoregram (Fig. 3) separated three components. After several successive analyses using capillary-electrophoresis algorithms published by Agilent Technologies [16], we found that the identified components were anionic and were classified as acidic proteins.

Isoelectric focusing on PAAG plates containing ampholines in the pH range 3.5-10 also confirmed the capillary-electrophoresis results. The peroxidase isospectrum in leaves of An-Bayaut-2 and C-4727 consisted mainly of acidic isoforms with pI 3.5 and 4.7. Basic isoforms with pI 8.0 and 7.8 were present in insignificant amounts. The activity of acidic isoperoxidases increased upon infection with pathogen. It was greater for An-Bayaut-2. Possible involvement of anionic plant

peroxidase isoforms in lignification of the cell wall has been reported [17, 18]. It was shown [17] that peroxidase isoforms with pI greater than 3.7 involved in suberinization of damaged tissues.

By comparing our results for peroxidase activity with the literature data, it can be concluded that An-Bayaut-2 is resistant to wilt whereas C4727 is a poorly resistant plant.

Possible mechanisms of involvement of peroxidase isoforms in protective responses upon infection by pathogenic fungi have been proposed [10]. The viability of the organism is in no way determined by peroxidases alone in complicated self-regulating systems such as plants with a large number of biopolymers and supramolecular structures and formations. However, peroxidases can convey enough information about the physiological condition of the plants that they can act as a criterion of the resistance to stresses.

Thus, acidic isoforms of peroxidase in wild and cultivated plants of the family Malvaceae were shown to be involved in protection of the plants against the phytopathogen *V. dahliae*.

EXPERIMENTAL

We studied four species of plants from the family Malvaceae, *M. sylvestris* L., *M. meluca* L., *H. esculentus* L., and *A. rosea*, in addition to various species and varieties of cotton from this same family, *G. klotzschianum* Andress, *G. raimondii* Ulbr., and two industrial varieties of *G. hirsutum*, An-Bayaut-2 and C-4727.

Infection of plants was performed by innoculating soil with fungal conidia in pots at temperatures below 25°C or through the root system of plants by placing them in vessels with solutions of the fungi. Plants in field conditions at temperatures above 25°C were innoculated with pathogen through capillaries fixed into the plant stems. The concentration of *V. dahliae* was 10^6 conidia/mL. Samples were collected 1 and 5 d after innoculation.

Isolation of Peroxidase from Malvaceae Leaves. The isolation medium was phosphate buffer (0.01 M, pH 6.0) in KNO_3 (0.1 M) + DEAE-Sephadex calculated for 1:5 buffer and 10:1 DEAE-Sephadex.

The isolation procedure consisted of grinding leaves in liquid N_2, homogenizing in a mortar with buffer, extracting for 30 min on a stirrer, filtrating, and precipitating protein with $(NH_4)_2SO_4$ (80% saturation). The floating precipitate was dissolved in distilled water and dialyzed against distilled water. The dialysate was lyophilized and used in the investigations.

Peroxidase activity was determined spectrophotometrically by the literature method [19]. Electrophoretic separation of peroxidases used basic (pH 8.9) PAAG (7.5%) [20] and benzidine for development.

Elution of proteins from gel was carried out by extracting protein from gel void by diffusion for 1 d at 37-40°C using sodium phosphate buffer (0.1 M, pH 6.0) with added sodium dodecylsulfate (DDS-Na) solution (1%). The suspension was ground in a glass homogenizer during the extraction. When the extraction was finished, gel was removed by centrifugation. DDS-Na and dye were removed by precipitating protein and washing first with acetone mixed with HCl (0.1 M, 6:1) and then pure acetone. The precipitated protein was dried by lyophilization.

Capillary electrophoresis was carried out on an Agilent Technologies instrument by capillary zone electrophoresis, which is most often used for peptide and protein analysis [21]. The conditions were capillary length 64.5 cm, inner diameter 50 μm; working buffer 20 mM borate; pH 9.3; injection 200 mbar; cassette temperature 25°C; 30 kV potential, positive polarity.

ACKNOWLEDGMENT

The work was supported financially by the USDA/ARS (project P-115) and the Republic of Uzbekistan Center for Science and Technology (project A-11-206).

REFERENCES

1. B. A. Rubin, *S'kh. Biol.*, **5**, 643 (1969).
2. B. A. Rubin, V. A. Aksenova, and O. N. Kozhanova, *Dokl. Akad. Nauk SSSR*, **210**, No. 2, 485 (1973).
3. B. A. Rubin and E. R. Kartashova, *Vesti S'kh. Nauki*, 3 (1970).

4. F. Passardi, C. Penel, and C. Dunand, *Trends Plant Sci.*, **9**, 534 (2004).
5. M. Y. Graham and T. Z. Graham, *Plant Physiol.*, **97**, 1445 (1991).
6. S. E. Melon, *Plant Physiol.*, **95**, 14 (1991).
7. K. E. Espellic, V. R. Francheschi, and P. E. Kollattukudy, *Plant Physiol.*, **81**, 487 (1986).
8. D. Kuch, *Immunization of Melons Against Fungal, Bacterial, and Viral Diseases. Infectious Diseases: Physiological and Biochemical Principles* [in Russian], Agropromizdat, (1985), pp. 150-168.
9. P. Seevers, J. Daly, and F. Catedral, *Plant Physiol.*, **48**, 13 (1971).
10. I. M. Savich, *Usp. Sovrem. Biol.*, **107**, 406 (1989).
11. V. A. Andreeva, *Peroxidase Enzyme* [in Russian], Nauka, Moscow (1988).
12. K. Tsachiya and K. Hirata, Ann. Meeting Phytopathol. Soc. Japan, 27 (1972).
13. J. W. Hendrix, R. D. Bennett, and E. Heltmann, *Microbios*, **5**, 31, 11 (1970).
14. C. Malier, C. P. S. Taylor, S. Sheier-Mucullo, and J. C. P. Smith, *Arch. Biochem. Biophys.*, **163**, 32, 671 (1974).
15. C. H. Beckman, *Annu. Rev. Phytopathol.*, **2**, 231 (1964).
16. Agilent Technologies, pub. No. 5988-6616 EN, 2002.
17. L. M. Lagrimini and S. Rothstein, *Plant Physiol.*, **84**, 438 (1987).
18. H. Grisebach, *The Biochemistry of Plants*, E. E. Conn, ed., Academic Press, New York (1986), Vol. **7**, 451.
19. A. N. Boyarkin, *Biokhimiya*, **16**, 7, 352 (1951).
20. B. N. Davis, *Ann. Acad. Sci. M.*, **2**, 404 (1964).
21. Agilent Technologies, pub. No. 5091-9062 EN, 2002.