Biochemistry (Moscow), Vol. 68, No. 1, 2003, pp. 111-115. Translated from Biokhimiya, Vol. 68, No. 1, 2003, pp. 133-138.

"Chitin-Specific" Peroxidases in Plants

I. V. Maksimov*, E. A. Cherepanova, and R. M. Khairullin

Institute of Biochemistry and Genetics, Ufa Scientific Center of the Russian Academy of Sciences, pr. Oktyabrya 69, Ufa, 450054 Russia; fax: (3472) 35-6100; E-mail: phyto@anrb.ru

Received December 17, 2001
Revision received February 5, 2002

Abstract—The activity of various plant peroxidases and the ability of their individual isoforms to bind chitin was studied. Some increase in peroxidase activity was observed in crude extracts in the presence of chitin. Activated peroxidases of some species fell in the fraction not sorbed on chitin and those of other species can bind chitin. Only anionic isoperoxidases from oat (*Avena sativa*), rice (*Oryza sativa*), horseradish (*Armoracia rusticana*), garden radish (*Raphanus sativus* var. *radicula*), peanut (*Arachis hypogaea*), and tobacco (*Nicotiana tabacum* Link et Otto) were sorbed on chitin. Both anionic and cationic isoforms from pea (*Pisum sativum*), galega (*Galega orientalis*), cucumber (*Cucumis sativus*), and zucchini (*Cucurbita pepo* L.) were sorbed on chitin. Peroxidase activation under the influence of chitin was correlated to the processes that occur during hypersensitive reaction and lignification of sites, in which pathogenic fungus penetrates into a plant. The role of chitin-specific isoperoxidases in inhibition of fungal growth and connection of this phenomenon with structural characteristics of isoperoxidases are also discussed.

Key words: peroxidase, isoenzymes, chitin, activation, sorption

During pathogenesis plants increase the level of lignin, which is the most effective barrier against phytopathogen invasion [1-4]. This suggestion derives from the fact that lignin-like material accumulates in the sites in which a pathogenic fungus penetrates into plant cells [3-5]. The exact mechanism of this phenomenon is unclear, but one can suppose that phytopathogenic organisms carry some substances on their cell surface that attract plant enzymes involved in lignin biosynthesis. Chitin and/or glucans abounding in cell walls of pathogenic fungi are possible candidates for the role of such substances [6].

Plant lectins and chitinases are proteins that directly interact with chitin [7-9], but they do not participate in reactions of lignin production. Peroxidase is an enzyme that quickly responds to various stress agents and actively joins lignification processes [10, 11]. Its activity many times increases in plant tissues infected by pathogens [12-14]. This is characteristic of isoperoxidases associated with cell wall [2, 5]. Data have been reported on the ability of peroxidases to join effectively the intermolecular link processes encompassing various plant cell wall components, such as cellulose, callose, xylans, and pectins [15-17]. However, the broad substrate specificity and multiplicity of peroxidase isoforms obscure the role of this enzyme in plant development and adaptation to a hostile environment.

We previously reported that anionic wheat peroxidases can bind both chitin and cells of the fungus *Tilletia caries* Tull. causing bunt disease [18]. However, it remained unknown how this feature is characteristic of peroxidases from other plant species. So, the problem we aimed to solve in the presented study was to find some chitin-binding isoperoxidases in various plant species. In our opinion, the solution to this problem could elucidate mechanisms protecting plants against invasion of phytopathogenic fungi.

MATERIALS AND METHODS

Four-day plantlets of oat (*Avena sativa* L., cultivar Skakun), rice (*Oryza sativum* L., VIR K-4573), garden radish (*Raphanus sativus* var. *radicula*, cultivar Zhara), pea (*Pisum sativum* L., cultivar Chishminskii), peanut (*Arachis hypogaea* L.), galega (*Galega orientalis* Lam.), tobacco (*Nicotiana tabacum* Link et Otto), cucumber (*Cucumis sativus* L., cultivar Libella), zucchini (*Cucurbita pepo* L., cultivar Souvenir), rhizomes of horseradish (*Armoracia rusticana* Gaertn., Mey. et Scherb), and etiolated plantlets of potato (*Solanum tuberosum* L., cultivar Nevskii) were chosen for study.

* To whom correspondence should be addressed.

0006-2979/03/6801-0111$25.00 ©2003 MAIK "Nauka/Interperiodica"

Chitin (Fluka, Switzerland) was ground in a grain mill, soaked in 2 M HCl for 2 h at room temperature, and washed with water. Then the chitin was soaked in 5-6 changes of 1 M NaOH for 2-3 h at 96°C in a water bath. Then the sorbent was washed with water till neutral pH. A chromatography column (2 × 6 cm) was filled with chitin suspension and equilibrated with 0.01 M phosphate buffer, pH 6.0.

Plant samples were treated with cold acetone and extracted as previously described [19]. Extract was applied onto the column containing chitin at room temperature. The column was washed until absorption at 280 nm was no more than 0.005, and then proteins bound to chitin were eluted with 1 M NaCl. The flow rate was 30 ml/h.

Peroxidase activity in chromatographic fractions was determined using a microassay protocol. The wells of Linbro flat-bottom polystyrene plates (Flow Laboratories, United Kingdom) were filled with 0.075 ml of 0.1 M phosphate buffer, pH 5.8, containing 0.025 ml of enzyme sample, 0.025 ml *o*-phenylenediamine (0.5 mg/ml), and 0.025 ml H_2O_2. Color development was stopped with 0.050 ml 2 M H_2SO_4. The plates were scanned on an IFCO-2 scanner (Russia) at 492 nm. Protein concentration was determined by the method of Bradford [19].

Isoelectric focusing was performed on an isoelectrofocusing apparatus (Hiiu-Kalur AS, Estonia) according to the manufacturer's recommendations in 7% polyacrylamide gel containing 10% glycerol and 1.5% ampholines, pH 3.5-10 (LKB, Sweden). Anode and cathode solutions were, respectively, 0.02 M H_3PO_4 and 0.1 M NaOH. Human hemoglobin (p*I* 7.0) was used as a marker [20]. Before application on a gel, samples were matched by protein content. Isoperoxidase activity in the gel was detected with 0.01% 3,3′-diaminobenzidine plus 0.005% H_2O_2 in 0.1 M phosphate buffer, pH 6.0. We designated isoenzymes positioned in gel between anode and the marker protein as anionic and between cathode and the marker protein as cationic.

RESULTS

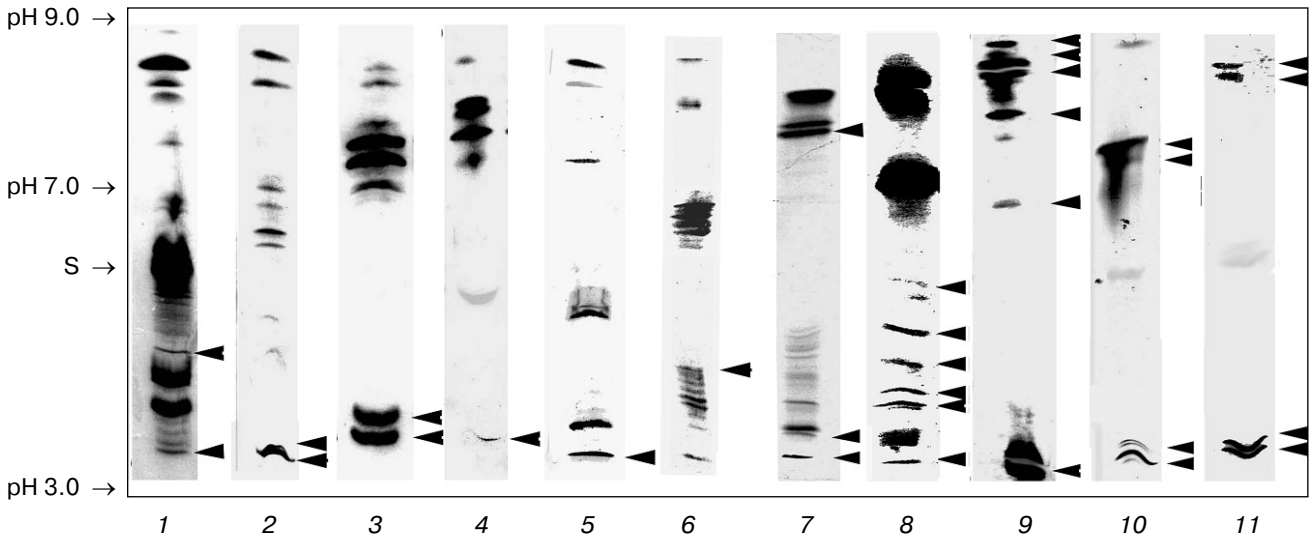
Data presented in the table show that peroxidase activity in the tested plants increases after exposure to chitin. Interestingly, in some species such increase was observed in a fraction not sorbed to chitin, and in others also in a fraction eluted from chitin with 1 M NaCl. In particular, potato peroxidase activity four times increased (table) in the fraction not bound to chitin, owing to anionic isoperoxidase.

Peroxidase activity in a fraction eluted from chitin gives evidence for the ion-exchange sorption of some peroxidase isoforms of studied species, as reported for wheat peroxidase [18]. So, we isoelectrofocused the protein samples after chromatography (figure). Note that extremely anionic isoperoxidases with p*I* near 3.0-3.5 were only sorbed on chitin (figure). Unlike above-mentioned plant species, this isoperoxidase from potato was not sorbed on chitin, but chitin many times increased the activity. It was another anionic peroxidase with higher p*I* that was sorbed on chitin.

The figure shows the isoelectrofocusing pattern of peroxidases isolated from the plantlets of rice, oat, garden radish, pea, peanut, galega, tobacco, cucumber, zucchini,

Effect of chitin on the peroxidase activity in extracts from various plants

Species	Peroxidase activity, arbitrary units per mg protein			Number of isoperoxidases	
	crude extract	fraction not sorbed on chitin	salt eluent	total in crude extract	sorbed on chitin
Soft wheat	10.5 ± 1.2	4.3 ± 0.5	11.0 ± 3.0	9	1
Oat	3.6 ± 0.5	3.1 ± 0.1	11.2 ± 2.5	10	2
Rice	1.7 ± 0.2	4.9 ± 0.6	0.5 ± 0.1	17	2
Horseradish	7.0 ± 1.0	19.2 ± 2.3	2.0 ± 0.1	8	2
Garden radish	9.0 ± 1.0	11.3 ± 2.0	2.9 ± 0.2	6	1
Potato	1.9 ± 0.2	6.8 ± 1.1	1.6 ± 0.1	18	1
Tobacco	2.4 ± 0.9	9.0 ± 0.9	13.7 ± 2.0	6	1
Peanut	8.2 ± 1.1	1.3 ± 0.1	6.1 ± 0.9	12	6
Pea	3.8 ± 0.3	2.9 ± 0.5	36.2 ± 3.9	11	6
Galega	3.9 ± 0.5	4.3 ± 0.1	4.2 ± 0.1	11	6
Cucumber	9.8 ± 0.5	1.9 ± 0.1	13.5 ± 0.9	5	4
Zucchini	6.2 ± 0.6	1.7 ± 0.1	5.1 ± 0.8	4	4



Isoenzyme spectra of peroxidases isolated from plantlets of rice (*1*), oat (*2*), tobacco (*3*), garden radish (*4*), rhizomes of horseradish (*5*), etiolated plantlets of potato (*6*), pea (*7*), peanut (*8*), galega (*9*), cucumber (*10*), and zucchini (*11*). Arrows indicate "chitin-specific" anionic and cationic isoperoxidases; S, point of sample application onto the gel

etiolated plantlets of potato, and rhizomes of horseradish. As one can see, in plants belonging to the bean and gourd families (pea, peanut, galega, cucumber, zucchini), unlike the other plant species, cationic isoperoxidases can also be sorbed actively on chitin.

DISCUSSION

The activation of defense proteins in pathogenesis is suggested to be a systemic response involving a rather long multistep information transfer into the plant cell and *de novo* biosynthesis of pathogen response protein (PR-protein) [21]. Unlike other PR-proteins, peroxidase as a protein essential to the normal functioning of plant cells [22] is constitutively present in plants. As a rule, this enzyme is activated under the influence of stress agents, especially of biotic ones [10].

We found that peroxidase activity in all plants tested increased in the presence of chitin compared to the activity in crude extracts. This activation can occur not only in chitin-sorbed fraction, but also in the fraction not sorbed on chitin. These data confirm the possibility of peroxidase activation in the presence of polysaccharides [23]. This increase in activity is probably due to conformational change of the enzyme molecules [24] or protein clearing out from the extract various inhibitory proteins possessing higher affinity to chitin and remaining bound to it even after elution with 1 M NaCl.

Analysis of isoenzyme spectra of peroxidases sorbed on chitin revealed both resemblance and differences between plant species. In particular, almost in all species tested chitin bound anionic isoperoxidases, but in some cases (in plants belonging to the bean and gourd families) some cationic isoforms can also be sorbed on chitin.

The characteristic of peroxidases to be activated by chitin and to be sorbed on it suggests their involvement in processes underlying two reaction types providing the plant with protection against phytopathogens. The first type includes fast activation of the enzyme upon its contact with pathogen cell structures, as observed particularly for the rice, potato, and horseradish peroxidases interacting with chitin (table). Peroxidase activation by chitin is probably comparable with its activation in the hypersensitivity reaction. The latter is known to be one of the most powerful defense features of plants, in which the plant organism sacrifices a portion of infected cells thereby ridding the plant of phytopathogen [25, 26]. Such kind of reaction often develops when host plant cells are affected by avirulent populations of biotrophic pathogens [27]. The second reaction type is comparable with gradual accumulation of the enzyme molecules within a region of fungus location associated with the appearance of a peculiar "attracting" center in the form of chitin-containing phytopathogen structures. Peroxidase binding with chitin or its large oligomers can result initially in the decrease in concentration of motile enzyme molecules interacting with chitin in this site of the cell. This probably results in the influx of new enzyme molecules. Thus a peculiar flux is formed of isoenzymes to the site of fungus localization resulting in saturation of the chitin-containing phytopathogen mycelium surface with specific isoper-

oxidases [18, 28]. Hence, favorable conditions for lignification of the region of phytopathogen penetration and its localization in this site appear thereby in an incompatible pathogenic system [1, 3].

We cannot rule out the coupling of these phenomena. In this case, a necrosis occurs associated with generation of toxic free radicals in the tissues subjected to intensive colonization with fungus [29, 30]. A lignification of single pathogen structures occurs with the involvement of isoperoxidases able to sorb on phytopathogen cell wall in cells close to necrosis [28].

Hence, the outcome of the pathological process probably depends on the rate of peroxidase inactivation in infected cells and on the intensity of enzyme sorption on mycelium of chitin-containing pathogen. For instance, we have demonstrated lignin synthesis of greater intensity and manifold elevation of anionic "chitin-specific" isoperoxidase activities in wheat resistant to *Septoria nodorum* [2] and *T. caries* [31].

The role of some isoperoxidases in plant resistance to phytopathogens has been discussed for many years, but the contribution of each peroxidase in the formation of physiological processes that occur in plants in pathogenesis is not well defined. Anionic isoperoxidases are known to be substantially activated when a plant is damaged by a phytopathogen or insect [32, 33]. They are thought to be responsible for lignification of impaired plant tissues [33, 34]. It is notable that zucchini anionic isoperoxidases sorbed on chitin can interact with plant oligogalacturonides in the presence of Ca^{2+} as well [35].

Thus, we have demonstrated peroxidase activation on contact with chitin and a capability of "chitin-specific" isoperoxidases to concentrate themselves on chitin. As we can judge from our data, the sorption to chitin of "chitin-specific" isoperoxidases may occur by a mechanism other than typical ion exchange in nature, because both anionic and cationic plant peroxidases can bind this biopolymer. Wherein isoperoxidases possessing similar isoelectric points can display different affinity to chitin, enabling one to compare the "chitin-specific" isoperoxidase isoforms with monovalent plant lectins extensins remarkable for their high affinity to hemicellulose [15, 16]. They are functionally bound to plant cell wall and act as its modifiers. It is notable that proline-rich sites similar to those in extensins are found in some peroxidase isoforms isolated from *Scutellaria baicalensis* Georgi, family Fabaceae [36]. Some tomato isoperoxidases are known to be able to form complexes with typical cell wall extensin [16].

These data unveil peculiarities of plant peroxidases in regards to their interaction with polysaccharides, as well as the role of this phenomenon in regulation of plant defense mechanism against penetration of chitin-containing phytopathogens.

This study was supported by the Russian Foundation for Basic Research (grant No. 01-04-48495) and an Award of the VI Expert Competition Projects among Young Scientists of the Russian Academy of Sciences (grant No. 207).

REFERENCES

1. Ride, J. P. (1980) *Phys. Plant Pathol.*, **16**, 187-196.
2. Yarullina, L. G., Maksimov, I. V., and Yamaleev, A. M. (1997) *Mikol. Fitopatol.*, **31**, 65-69.
3. Cadena-Gomes, G., and Nicholson, R. L. (1987) *Phys. Mol. Plant Pathol.*, **31**, 51-67.
4. Inoue, S., Aist, J. R., and Marko, V. (1994) *Phys. Mol. Plant Pathol.*, **44**, 433-440.
5. Medeghini, B. P., Larenzini, G., Baroni, F. R., Nali, C., and Sgaili, E. (1994) *J. Phytopathol.*, **140**, 319-325.
6. Peberdy, J. F. (1989) *Biochemistry of Cell Wall and Membranes in Fungi* (Kuhn, P. J., ed.) Springer-Verlag, Berlin, pp. 5-21.
7. Barraqueta-Egea, P., and Schauz, K. (1983) *Z. Pflanzenkrankheiten Pflanzenschutz.*, **90**, 488-495.
8. Brambl, R., and Gade, W. (1985) *Physiol. Plant.*, **64**, 402-408.
9. Inui, H., Kosaki, H., Uno, Y., Tabata, K., and Hirano, S. (1991) *Agric. Biol. Chem.*, **55**, 3107-3109.
10. Savich, I. M. (1989) *Uspekhi Sovrem. Biol.*, **107**, 406-417.
11. Edreva, A. M. (1991) *Fiziol. Rast.*, **38**, 788-800.
12. Kerby, K., and Sommerville, S. (1989) *Phys. Mol. Plant Pathol.*, **35**, 329-337.
13. Kazan, K., Goulter, K. C., Way, H. M., and Manners, J. M. (1998) *Plant Sci.*, **136**, 207-217.
14. Caruso, C., Chilosi, G., Caporale, C., Leonardi, L., Bertini, L., Magro, P., and Buonocore, V. (1999) *Plant Sci.*, **140**, 87-97.
15. Brownleader, M. D., Ahmed, N., Trevan, M., Chaplin, M. F., and Dey, P. M. (1994) *Plant Peroxidase Newsletter*, **4**, 2-13.
16. Brownleader, M. D., Hopkins, J., Mobasheri, A., Dey, P. M., Jakson, P., and Travan, M. (2000) *Planta*, **210**, 668-676.
17. Bradley, D. J., Kjellbom, P., and Lamb, C. J. (1992) *Cell*, **70**, 21-30.
18. Maksimov, I. V., Khairullin, R. M., Yamaleev, A. M., and Yamaleeva, A. A. (1995) in *Problems of Biotechnology* (Akhmetov, R. R, ed.) [in Russian], Bashkir State University, Ufa, pp. 120-127.
19. Ermakov, A. M., Arasimovich, V. V., and Yarash, N. P. (1987) *Biochemical Methods in Plant Research* [in Russian], Agropromizdat, Leningrad.
20. Blumenfeld, L. A. (1957) *Hemoglobin and Reversible Oxygenation* [in Russian], Academy of Sciences of the USSR, Moscow.
21. Tarchevsky, I. A. (2000) *Appl. Biochem. Microbiol. (Moscow)*, **37**, 441-455.
22. Gazaryan, I. G. (1992) *Biotechnology of Plant and Fungal Peroxidases*, in *Advances in Science and Technology. Biotechnology* [in Russian], Vol. 36, VINITI, Moscow.
23. Siegel, S. M. (1958) *J. Am. Chem. Soc.*, **79**, 1628-1632.
24. Keleti, T. (1986) *Basic Enzyme Kinetics*, Akadémiai Kiadó, Budapest.
25. Mani, B. M., and Metraux, J. P. (1998) *Ann. Botany*, **82**, 535-540.

26. Dangl, J. L., Dietrich, R. A., and Richberg, M. H. (1996) *Plant Cell*, **8**, 1793-1807.
27. Wojtaszek, P. (1997) *Biochem. J.*, **322**, 681-692.
28. Khairullin, R. M., Yusupova, Z. R., and Maksimov, I. V. (2000) *Fiziol. Rast.*, **47**, 108-113.
29. Apostol, I., Heinshtein, P. F., and Low, P. S. (1989) *Plant Physiol.*, **90**, 109-116.
30. Peng, M., and Kuc, J. (1992) *Phytopathology*, **82**, 696-699.
31. Khairullin, R. M., Yusupova, Z. R., and Maksimov, I. V. (2001) *Mikol. Fitopatol.*, **35**, No. 3, 47-53.
32. Repka, V., and Fisherova, I. (1998) *Biol. Plant.*, **40**, 605-615.
33. Espelie, K. E., and Kolattukudy, P. E. (1985) *Arch. Biochem. Biophys.*, **240**, 539-545.
34. Dowd, P. F., Lagrimini, L. M., and Nelsen, T. C. (1998) *Natural Toxins*, **6**, 241-249.
35. Penel, C., van Cutsem, P., and Grappin, H. (1999) *Phytochemistry*, **51**, 193-198.
36. Morimoto, S., Tateishi, N., Inuyama, M., Taura, F., Tanaka, H., and Shoyama, Y. (1999) *J. Biol. Chem.*, **274**, 26192-26198.