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PEROXIDASE ACTIVITY IN HEALTHY AND LEAF-RUST-INFECTED WHEAT LEAVES*

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Abstract—Peroxidase activity was determined in primary leaves of healthy and inoculated, near-isogenic wheat lines differing in susceptibility to the leaf rust pathogen, *Puccinia recondita* (isolate UN1-68B). Inoculated LR10(TC), which developed a low (X) infection type, had peroxidase activity 109% greater than healthy controls by 9 days post-infection. Thatcher, which developed a high (4) infection type, had peroxidase activities only 20–48% greater than healthy controls during the 2–9-day period after infection. Activity, similar in healthy leaves of both lines, increased with leaf age. Total buffer-soluble protein (trichloroacetic acid-precipitable) did not change as the disease developed in either line. Peroxidases from both healthy and inoculated LR10(TC) separated on a gel filtration column into two distinct molecular weight groups. The ratio of activities in these two groups did not differ between 9-day-rusted LR10(TC) and healthy tissue, although total peroxidase was considerably greater in infected tissues. This suggests that the two forms may be related. The low molecular-weight peroxidase fraction had an average molecular weight of near 35,000 (as determined by gel filtration), while the molecular weight of the other fraction was near 160,000. The physiological significance of the two molecular sizes is unknown.

INTRODUCTION

PEROXIDASE (E.C.1.11.1.7) activity frequently increases in plants infected by phytopathogens, and disease resistance and peroxidase activity often correlate closely.[1] Peroxidase activity increases in several plant hosts following rust infection,[2–8] with greatest increases in resistant-reacting tissues.[2–4,6,7] Peroxidase occurs widely in healthy plant tissues, but its precise physiological role is uncertain.[9] Peroxidase increases have been suggested as being directly responsible for disease resistance; mycelial growth of the stem rust fungus (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.) *in vitro* is inhibited by peroxidase.[6] However, neither total peroxidase activity[2,7] nor specific peroxidase isoenzymes[10] are causally related to resistance in wheat infected by this pathogen. Rather, peroxidase increases apparently result from a nonspecific incompatibility caused by a prior biochemical event in resistant reactions to the stem rust fungus.[4] Whether the results apply to other incompatible host-rust

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combinations, or what the mechanism of change in peroxidase activity is remain unanswered questions. We report here studies on leaf peroxidases from healthy wheat leaves and the changes that occur following infection by the leaf-rust fungus, *Puccinia recondita* Rob. ex Desm.

RESULTS

Peroxidase Activity in Cell-free Extracts

Table 1 shows the change in peroxidase activity with time (after leaf rust inoculation) in extracts from susceptible-reacting and resistant-reacting wheat leaves, compared with activity in healthy controls. Peroxidase levels were similar in extracts of healthy Thatcher and LR10(TC) leaves, and increased in both wheat lines with leaf age. Total activity in LR10(TC) eventually doubled. Peroxidase activity in the inoculated susceptible-reacting Thatcher varied between 20 and 48% higher than in the healthy control. Increases were much greater in the resistant-reacting LR10(TC), with a maximum of 109% over the healthy controls 9 days after inoculation.

TABLE 1. PEROXIDASE ACTIVITY* IN CELL-FREE EXTRACTS OF HEALTHY AND INOCULATED THATCHER (SUSCEPTIBLE) AND LR10(TC) (RESISTANT) WHEAT LEAVES

Days after inoculation	Thatcher			LR10(TC)		
	Healthy (activity)*	Inoculated (activity)*	Increase %	Healthy (activity)*	Inoculated (activity)*	Increase %
2	14·5	19·4	34	15·2	22·0	45
3	16·0	21·1	33	17·8	23·7	33
4	16·7	20·1	20	15·7	26·8	71
6	17·6	26·1	48	19·9	36·9	85
9	22·6	27·1	20	22·5	47·1	109

Data are averages of 2 assays each of 2 extracts of 8–11 primary leaves (1 g fresh wt./3 ml buffer). The average variation between duplicate samples was ± 4·3% and did not exceed ± 9·7%.
* Expressed as μmoles H_2O_2 consumed/min/ml.

Peroxidase activity was determined every 0·5 pH value from 4·0 to 9·0 for both healthy and inoculated leaves from LR10(TC). In both cases, maximal activity was at pH 6·0. There was no shift in the peroxidase pH optimum with disease development, so all assays were run at pH 6.

Gel Filtration Chromatography of Wheat Leaf Extracts

Sephadex G-100 chromatography of extracts from healthy and inoculated LR10(TC) gave elution patterns of peroxidase activity as shown in Fig. 1. Two peroxidase peaks were found for each extract. The high molecular weight peroxidases, which eluted after the Blue-Dextran peak (Fraction 31), contained 16 and 14% of the total activity for extracts of healthy and rusted tissue, respectively. Distribution of activity between high molecular-weight and low molecular-weight forms is, therefore, about the same for healthy and infected tissue; however, extracts from infected tissue, in this experiment, had about 85% more total activity than the healthy-leaf extract. The lower molecular-weight peak is asymmetrical, and probably contains peroxidases of slightly different size. Disc electrophoresis studies show several electrophoretically distinct components.

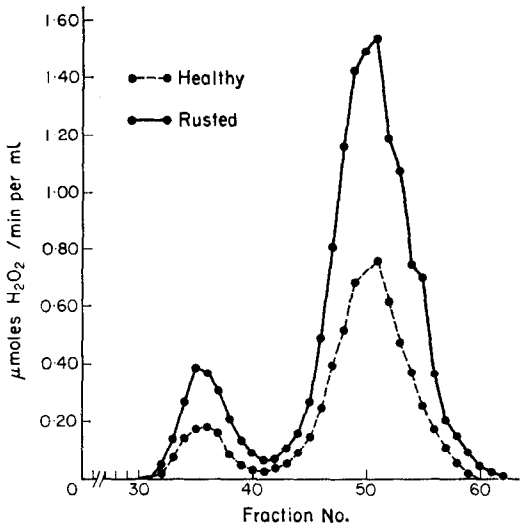

FIG. 1. FRACTIONATION OF PEROXIDASE ACTIVITY FROM HEALTHY AND 9-DAY-RUSTED LR10(TC) WHEAT LEAVES ON A SEPHADEX G-100 COLUMN.
Crude cell-free extract (0·5 ml) of each sample was applied, and 2·3 ml fractions were collected at an elution rate of 14·0 ml/hr at 1–5°. The column equilibration and elution buffer was 0·1 M Tris HCl, pH 8·5, containing 0·5 M NaCl. The column bed was 2·5 × 39 cm.

The nature and physiological significance of the two widely different peroxidase sizes are uncertain. Altering ionic strength (either 0 or 0·5 M NaCl) in the column buffer did not significantly modify the ratios of the two peroxidase peaks. Other enzymes, e.g. chlorophyllase,[11] which have high and low molecular weight forms, show altered molecular weight distributions as ionic strength is changed.

A molecular weight determination for low molecular weight wheat leaf peroxidases using our buffer and the procedure of Whitaker[12] gave values near 35,000, which may be an average for the several components of the low molecular-weight group.

Horseradish peroxidase, on our column, gave a molecular weight of 50,000, only slightly higher than that reported by Andrews[13] and higher than the accepted 40,000. Andrews reasoned that glycoproteins may have a more expanded structure than other proteins, and that gel filtration might overestimate their molecular weight. If low molecular weight wheat leaf peroxidase is a glycoprotein, we might have overestimated its molecular weight as well. However, in all our experiments, horseradish peroxidase eluted somewhat earlier than low molecular weight wheat leaf peroxidase. The high molecular weight peroxidase eluted earlier than bovine serum albumin, and later than wheat leaf catalase. Preliminary experiments employing gel filtration on agar beads (Biogel A-1·5 m) indicate the high molecular weight wheat leaf peroxidase has a molecular weight near 160,000.

Soluble Protein and Peroxidase Concentrations in Healthy and Infected Leaves

Soluble protein in the cell-free extract was determined after precipitation with trichloroacetic acid. The results in mg protein/ml of extract at 2, 3, 4, 6 and 9 days, respectively, were: healthy Thatcher, 3·6, 4·2, 4·7, 4·8 and 4·8; inoculated Thatcher, 3·7, 4·1, 4·3, 4·3

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and 4·2; healthy LR10(TC), 3·9, 4·1, 4·8, 5·0 and 5·3; inoculated LR10(TC), 4·0, 4·5, 4·7, 4·9 and 4·9. We conclude that no pronounced change has occurred in the amount of soluble leaf protein present after infection with the leaf rust fungus for either resistant or susceptible wheat. Soluble protein in all samples was comparable at any given age. Peroxidase activity was expressed in units/ml because units/mg protein introduced slightly more variability without changing the overall conclusions.

DISCUSSION

The resistant response to isolate UN1-68B in LR10(TC) was characterized by about twice the increase in peroxidase activity of the susceptible response in its near-isogenic counterpart, Thatcher. That pattern also has been observed in several near-isogenic lines differing in resistance or susceptibility to stem rust in wheat,[4,7] although the response in stem-rust-resistant wheat tissues[6,7] appears to be greater. However, we do not yet know how characteristic this peroxidase response is of wheat having other leaf rust resistance genes. The use of other environments, rust isolates, levels of infection, or enzyme substrates might also result in different assayable peroxidase responses following infection.

The major increase in peroxidase activity following infection in LR10(TC) occurs when there is little change in total buffer-soluble protein. This peroxidase increase may represent a derepression of genes for its synthesis, or its release from a bound form, or some other mechanism of activation. Further studies on the increased peroxidase activity may clarify the general mechanism of enzyme responses in resistant-reacting tissues. However, as with peroxidase increase in response to stem rust infection,[2,4,7,10] peroxidase itself may not explain resistance.

The appearance of two distinct molecular weight groups for wheat leaf peroxidase has not been previously reported. Purified horseradish peroxidase gives a single peak and so does a *Lens* root peroxidase.[14] However, *Lens*,[15,16] pea,[16] and sorghum[17] peroxidases have been separated into both high- and low-molecular weight components, although molecular weights were not determined, except for the low molecular weight sorghum peroxidase.

Other enzymes which separate into high- and low-molecular weight components include chlorophyllase from sugar-beet leaves,[11] malate dehydrogenase from bean leaves,[18] and phosphatases from wheat leaves.[19] It is not always clear whether these forms reflect *in vivo* enzymes or are simply artifacts of isolation. High- and low-molecular weight forms might exist in the cell, but aggregation or disaggregation during the extraction process or binding to structural polymeric elements might equally well account for multiple molecular weight forms. Simple aggregation from ionic interaction seems unlikely, because varying the ionic strength in the Sephadex G-100 column did not significantly change the proportions of the two peroxidase peaks. As the ratio of the two peaks did not change during the relative doubling of activity in resistant-reacting tissues over healthy leaves, a relationship within the tissue is suggested. However, the relative activity in the two fractions throughout the infection cycle has not been determined. More direct evidence comparing these two molecular weight fractions must be obtained before an interpretation of their role

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can be made. Also, peroxidase zymograms from wheat leaf extracts should be reexamined to determine if molecular weight differences are superimposed on electrophoretic differences.

EXPERIMENTAL

Host and pathogen. Primary leaves were used from *Triticum aestivum* L. em Thell. var. Thatcher or LR10(TC) wheat seedlings grown in vermiculite and irrigated with nutrient solution.[20] Plants were kept in environmental chambers with a 23–26° photoperiod of 14 hr (18,500–20,000 lx from incandescent plus VHO cool-white fluorescent lamps) and a 16–18° dark period of 10 hr. Relative humidities averaged about 70%.

Seedlings were inoculated with *Puccinia recondita* Rob. ex Desm. isolate UN1-68B (ATCC PR66) 7–8 days after planting, then sprayed lightly with water, and incubated overnight in high humidity. The two near-isogenic lines exhibited different infection types. Thatcher (C.I. 10003) showed a 4 (high) infection type, while LR10(TC) (R.L. 6004), carrying the *Lr10* resistance gene, showed an X (low) infection type. Thatcher leaves averaged 30 pustules/cm^2. Counts were not made on LR10(TC).

Extraction of primary leaves. Leaves (1 g fresh wt.) were ground with a cold mortar and pestle, in 3 ml of 0·1 M, pH 8·5, Tris HCl buffer containing 0·5 M NaCl. We found that high pH and ionic strength were conducive to more complete extraction of wheat leaf peroxidase, as had previously been observed for extraction of bean leaf peroxidase.[21] Adding 0·5 M NaCl to H_2O or to 0·1 M, pH 8·5, tris buffer enhanced peroxidase extraction from healthy wheat leaves by + 54% or + 29%, respectively. When 0·1 M buffers containing 0·5 M NaCl were compared, pH 5·5 acetate, pH 7 phosphate, and pH 10 Tris were 80, 95, and 93% as effective as pH 8·5 Tris HCl buffer containing 0·5 M NaCl. The Tris HCl buffer, pH 8·5, containing 0·5 M NaCl was, therefore, used in all subsequent extractions. After being ground, samples were centrifuged at 37,000 *g* and 3° for 15 min. The supernatant fluid was stored at 0–5° and aliquots were assayed for peroxidase within 2–4 hr. The remainder of the supernatant fluid was frozen for later chromatographic analyses.

Peroxidase activity. Guaiacol was used as donor, and the absorbance change at 470 nm was recorded on a strip chart. Initial rates were obtained from the linear portion of the curves. All reactions were run at 28°. The reaction mixture contained 540 μmoles phosphate buffer, pH 6·0, 5·4 μmoles guaiacol, 2·7 μmoles H_2O_2, and 0·5 ml appropriately diluted enzyme or distilled water, in a final volume of 2·7 ml. The reaction was initiated by adding the H_2O_2 with stirring. H_2O_2 concentration was determined spectrophotometrically, assuming $\epsilon_{250nm} = 0{\cdot}0252\ cm^{-1}mM^{-1}$. Activity was expressed as μmoles H_2O_2 consumed/min/ml of undiluted enzyme, using the relation[22] 60 μM $H_2O_2 = 0{\cdot}4\ A_{470nm}cm^{-1}$. For our reaction volume of 2·7 ml, 1·0 $A_{470nm} = 0{\cdot}4$ μmole H_2O_2.

Protein. Buffer-soluble protein was first precipitated in 5% (final conc.) of trichloroacetic acid (TCA). The precipitate was washed once with 5% TCA, dissolved in 1·0 N NaOH solution, and assayed by the method of Lowry *et al.*[23] Bovine serum albumin was used as the standard.

Gel filtration. (a) A 2·5 cm dia. × 39 cm column bed of Sephadex G-100 (40–120 μ) was equilibrated with 0·1 M, pH 8·5, tris HCl buffer that contained 0·5 M NaCl. Samples (4 ml or less) in 10% sucrose were applied to the top of the column. All separations were done at 1–5°. Six fractions per hr were collected at a flow rate of 2·9 ml/hr/cm^2, maintained by a Cole–Parmer peristaltic pump. Mol. wts were estimated from a V_e/V_o plot against log mol. wt.,[12] using Blue Dextran (V_o), bovine serum albumin (67,000) and horse heart myoglobin (17,000).

(b) A 1·0 cm dia. × 112 cm column bed of Biogel A-1·5 m agar beads was equilibrated with 0·1 M NaOAc buffer, pH 6·0, containing 0·4 M NaCl, and operated in reverse-flow mode at 5·1 ml/hr (6·5 ml/hr/cm^2). The water-jacketed column was maintained at 8° and buffer was pumped as in (a) above. Mol. wt. standards in addition to those above were bovine thyroglobulin (670,000), bovine liver catalase (248,000), lactoperoxidase (82,000) and cytochrome c (12,400).

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Key Word Index—Triticum vulgare; Gramineae; peroxidase; rust infection; isoenzymes.