

Purification of an Infection-Related, Extracellular Peroxidase from Barley¹

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ABSTRACT

Increases in two extracellular peroxidases were observed following inoculation of barley (*Hordeum vulgare* L.) with the powdery mildew pathogen (*Erysiphe graminis* DC.: Fr. f. sp. *hordei* Em. Marchal). The more prominent isozyme, P8.5, was purified from intercellular wash fluids by acetone precipitation, ion-exchange chromatography, isoelectric focusing, and gel filtration. Purified P8.5 is a heme-containing, glycoprotein with a *M_r* of 35,000. It has eight cysteine residues. A highly specific, high-titer antiserum to deglycosylated P8.5 was produced.

Peroxidase isozymes, which are induced following inoculation with pathogens, are thought to be involved in the deposition of lignin-like compounds at sites of attempted penetration and wounding (6). The lignin-like deposits are assumed to provide a barrier to cell-wall degradation and fungal penetration of host tissue and, thereby, contribute to host defenses (27). It has also been suggested that peroxidases may cross-link phenolic compounds into plant cell-wall polymers and into callose-containing papillae at infection sites (5). Others have proposed that toxic radicals capable of inhibiting pathogen growth are produced extracellularly via peroxidase action (4).

In barley, the cytological responses most commonly correlated with the expression of resistance to the powdery mildew pathogen are the formation of papillae and hypersensitive necrosis (10, 14). In addition, various groups have reported that the deposition of autofluorescent, possibly lignin-like compounds in the wall, papillae, and host cytoplasm adjacent to infection sites is correlated with the inhibition of pathogen ingress (2, 30). Several of these responses can be observed in compatible host/pathogen interactions and in wounded plants (23).

The apoplast of the primary leaf of 7-d-old barley (*Hordeum vulgare* L.) contains at least six peroxidase isozymes as determined by IEF³ (11). Inoculation with *Erysiphe graminis*

f. sp. *hordei*, the causal agent of powdery mildew of barley, results in a 16-fold increase in extracellular peroxidase activity within 3 d of inoculation. The increased activity is a consequence of an increase in two isozymes with pI values of 8.5 and 5.2, which we have named P8.5 and P5.2, respectively (11).

Our long-term goal is to test the hypothesis that the pI 8.5 and pI 5.2 peroxidase isozymes are involved in the deposition of lignin-like compounds in papillae and in cross-linking phenolic or phenylpropanoid compounds into preexisting cell-wall polymers. As a step toward this goal, we report the purification of P8.5.

MATERIALS AND METHODS

Source of Biological Materials and Growth Conditions

The barley (*Hordeum vulgare* L.) line CI-16137 (Alg R) was obtained from Dr. J. G. Moseman (Beltsville, MD). The CI-16137 line carries the *Ml-a1* allele conditioning resistance to race CR3 of the powdery mildew pathogen, *Erysiphe graminis* DC.:Fr. f. sp. *hordei* Em. Marchal (20). Race CR3 was maintained on susceptible barley lines in growth chambers (11).

Protein Assays, Enzyme Activities, and Electrophoresis

Peroxidase activity was determined with guaiacol as the substrate (11). The *R_z* value was determined by calculating the ratio of the absorbance of the Soret band (405 nm) over the total protein absorbance (280 nm) as measured with a Beckman DU-70 spectrophotometer (29). The protein concentration of each sample was determined in triplicate with Bio-Rad reagents by the method of Bradford (3). Bovine gamma globulin was used as a protein standard.

Peroxidase isozymes were detected in nondenaturing IEF gels (pH 3.5–9.5 PagPlate, LKB, or pH 3.0–9.0 PhastGel, Pharmacia) with 3-amino-9-ethylcarbazole as the substrate (11). SDS-PAGE was carried out on 10% (w/v) acrylamide discontinuous gels (15). Protein samples were adjusted to 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 60 mM DTT, 12% (w/v) sucrose, and 0.05% (w/v) bromophenol blue and boiled for 5 min before loading onto the gel. Proteins were detected by silver staining (19).

Peroxidase Purification

For the induction of infection-related peroxidases, 7-d-old barley seedlings were inoculated with conidia of *E. g. hordei*,

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³ Abbreviations: IEF, isoelectric focusing; CMC, carboxymethyl cellulose; IWF, intercellular wash fluid; *R_z*, Reinheitszahl; pI, isoelectric point; HF, hydrofluoric acid.

race CR3 (11). The IWF was collected from the primary leaves 7 d after inoculation following the procedure of Kerby and Somerville (11). One modification of the original protocol was that leaves were infiltrated with water for 30 min (16). The protein solution obtained from the IWF was brought to 0.02 M sodium acetate (pH 5.0) and loaded onto a CMC (preswollen, microgranular, Whatman CM52) column (1.2 × 10 cm) that had been equilibrated with four volumes of 0.02 M sodium acetate (pH 5.0). The column was washed with 0.02 M sodium acetate (pH 5.0) until peroxidase activity was no longer detected in the void volume (usually about 20 mL). The column was then washed with 15 mL each of 0.06 M and 1.0 M sodium acetate (pH 5.0). The effluent from each step was collected separately and dialyzed against H₂O. The peroxidase activity of each fraction was assayed, and the IEF profile of peroxidase isozymes for each fraction with peroxidase activity was determined on wide pH-range, preformed polyacrylamide gels (pH 3.5–9.5 PagPlate, LKB) (11).

Fractions from the CMC column containing P8.5 were adjusted to 1% ampholytes (BioLyte 3/10, Bio-Rad) in a final volume of 50 mL, and the proteins were separated in a Rotofor IEF cell (Bio-Rad) following the manufacturer's instructions. Each fraction was tested for peroxidase activity. The IEF profile of each peroxidase-containing fraction was determined with the PhastSystem (pH range 3.0–9.0, Pharmacia). Fractions with a similar peroxidase isozyme pattern were pooled, brought to 1 M NaCl, and dialyzed for at least 24 h against H₂O (2 × 5000 mL). This solution was reduced in volume to about 5 mL by ultrafiltration with a PM10 filter (Amicon). The peroxidase fraction was adjusted to 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl, loaded onto a Sephadex G-75 column (3.8 × 97 cm, Pharmacia), and eluted with 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl. Fractions of 4.5 mL were collected. The peroxidase profile of the guaiacol-active fractions was determined on IEF gels (pH 3.5–9.5 PagPlate, LKB). Fractions containing identical IEF profiles were pooled and dialyzed against H₂O. The volume was reduced by ultrafiltration with a PM10 filter (Amicon).

HF Deglycosylation

Purified P8.5 (2 mg) was deglycosylated with anhydrous HF (12) with the following modifications. A volume of 2 mL of anhydrous HF was added to the cold 10% (v/w) anhydrous methanol-protein solution. The solvolysis reaction was stopped by adding this solution to 15 mL of cold (4°C) H₂O. The reaction mix was dialyzed against H₂O (2 × 6000 mL) at 4°C for 24 h and then lyophilized. Deglycosylated protein was purified by reverse-phase HPLC on a μ Bondapak C₁₈ column (2 mm i.d. × 30 cm). The protein was eluted with the following mobile phase solvents: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. A linear gradient began at 100% A and increased from 0% B to 100% B over 60 min (0.25 mL/min). The absorbance of the eluate was monitored at 214 nm.

Mol Wt Determination

The M_r of the P8.5 peroxidase isozyme was estimated by both gel filtration and SDS-PAGE. Protein was applied to a

Sephadex G-75 column (3.8 × 97 cm, Pharmacia) equilibrated with a solution of 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl. Protein was eluted with the same buffer-salt solution. The reference polypeptides were BSA (M_r 68,000), ovalbumin (M_r 43,000), chymotrypsinogen (M_r 25,000), myoglobin (M_r 17,200), and Cyt *c* (M_r 12,500). The M_r of the peroxidase isozymes were also estimated by SDS-PAGE with low mol wt standards (Bio-Rad) as reference proteins.

Amino Acid Analysis

The amino acid content of P8.5 was determined at the Macromolecular Facility, Michigan State University, following the procedure of Spackman et al. (26).

Antiserum Production and Immunoblotting Methods

Antiserum against purified, deglycosylated P8.5 was produced in New Zealand White rabbits (7). Protein (0.1 mg) was dissolved in 1 mL of PBS and then emulsified with an equal volume of Freund's complete adjuvant (Difco). Two rabbits were given four subcutaneous injections of 0.25 mL each. Nine months later, the rabbits were given a second injection consisting of 0.1 mg of deglycosylated P8.5 emulsified in Hunter's TiterMax (CytoRx Corp.) and administered according to the manufacturer's instructions. The animals were bled 10 d after the final injection, and serum was recovered from the blood sample (7).

For western blot analysis, proteins separated by SDS-PAGE were transferred to nitrocellulose (7). The nitrocellulose blot was treated first with anti-P8.5 antiserum, diluted 1/30,000, and then with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antiserum (Kirkegaard and Perry, Gaithersburg, MD). Bromochloroindolyl-phosphate and nitroblue tetrazolium were used as substrates for the alkaline phosphatase reaction (7).

RESULTS

The IEF profile of the extracellular peroxidases obtained from primary leaf tissue of barley 7 d after inoculation with *E. g. hordei* is given in Figure 1, lane 1. Six prominent bands were observed reproducibly. Based on their pI values, the six bands have been designated P4.8, P4.9, P5.2, P7.7, P8.5, and P9.3.

The P8.5 peroxidase isozyme was purified in a four-step procedure from IWF. The yield and recovery after each of the purification steps is given in Table I. The values were obtained during the purification of P8.5 from 500 g fresh weight of leaf tissue. When the crude IWF fraction was applied to a CMC column, only P9.3 bound to the matrix (Fig. 1, lane 3). The P9.3 peroxidase eluted with the 0.06 M sodium acetate wash (Fig. 1, lane 2). Most proteins of the IWF, including P8.5, did not bind to this cation-exchange matrix. The 0.02 M sodium acetate eluate from the CMC column was loaded into a Rotofor apparatus. After separation, each of the 20 fractions was tested for peroxidase activity, and the IEF profile of every peroxidase-containing fraction was determined. Fractions with identical isozyme patterns were pooled. By this separation technique, the an-

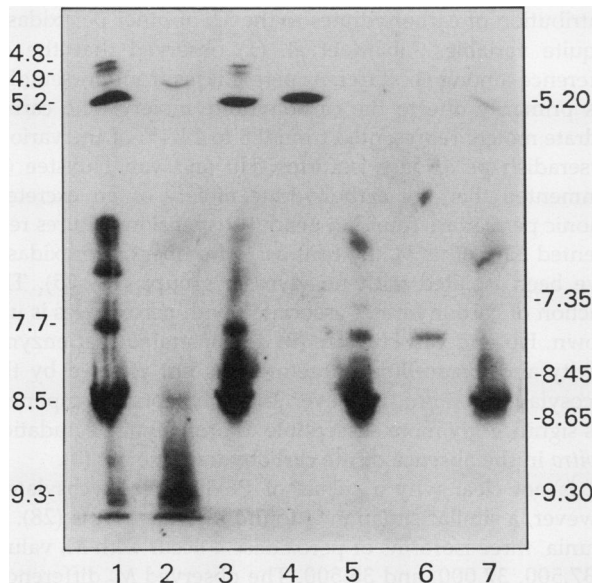


Figure 1. IEF profile of fractions collected during the purification of P8.5. Lane 1, Acetone-precipitated IWF. To the left of lane 1, the pI values of the six peroxidases that are reproducibly observed in IWF are given. Lane 2, 0.06 M sodium acetate eluate from the CMC column; lane 3, 0.02 M sodium acetate eluate from the CMC column; lane 4, acidic Rotofor fraction; lane 5, basic Rotofor fraction; lane 6, peroxidase peak 1 from the gel-filtration step; lane 7, peroxidase peak 2 from the gel-filtration step. The pI values of reference standards are listed to the right of lane 7. The gel was stained for peroxidase activity with 3-amino-9-ethylcarbazole.

ionic peroxidases were separated from the more basic peroxidases, including P7.7 and P8.5 (Fig. 1, lanes 4 and 5). The pooled sample containing P7.7 and P8.5 from the Rotofor separation was applied to a Sephadex G-75 gel-filtration column. Two peroxidase-active peaks were recovered. The peroxidase IEF profile of these peaks indicated that P7.7 eluted earlier than P8.5 (Fig. 1, lanes 6 and 7). A comparison of the elution volumes of these two peroxidases with the elution volumes of M_r standards indicated that P7.7 had a M_r of 65,000, whereas P8.5 had a M_r of 35,000 (data not presented).

To determine the total protein complexity at each separation step, fractions containing P8.5 were subjected to SDS-PAGE followed by silver staining (Fig. 2). When the IWF proteins were separated by SDS-PAGE, a number of bands were observed (Fig. 2, lane 1). With cation-exchange chro-

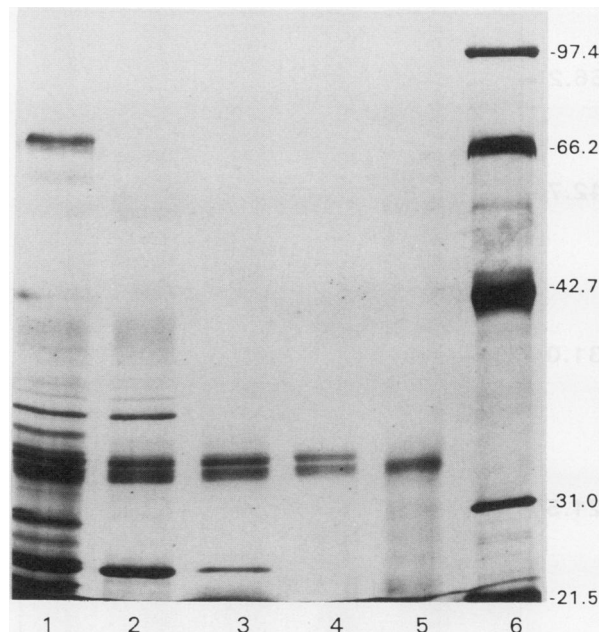


Figure 2. SDS-polyacrylamide gel of peroxidase-containing fractions obtained at each step during the purification of P8.5. Lane 1, Acetone-precipitated IWF; lane 2, 0.02 M sodium acetate eluate of the CMC column; lane 3, basic Rotofor fraction; lane 4, peroxidase peak 2 from the gel filtration column; lane 5, deglycosylated P8.5; lane 6, reference markers with their M_r ($\times 10^{-3}$). The gel was stained for total protein with silver.

matography, about 40% of the total protein, coeluted with P8.5 (Table I; Fig. 2, lane 2). After Rotofor separation, three polypeptide bands copurified with P8.5 activity (Fig. 2, lane 3). This represented 8% of the original protein (Table I). Selection for P8.5 activity after gel filtration resulted in two closely migrating bands (Fig. 2, lane 4) with estimated M_r values of 35,000 and 34,000. This fraction represented 5% of the total IWF protein (Table I). The Rz value of this fraction was 1.5.

To determine whether the M_r differences between the two P8.5 polypeptides were the result of differences in glycosylation, the polypeptides were chemically deglycosylated with anhydrous HF. A single band with a M_r of 33,900 was observed after deglycosylation (Fig. 2, lane 5).

A specific, high-titer antiserum was raised to deglycosylated P8.5. This antiserum exhibited a strong reaction with P8.5 (Fig. 3, lanes 2, 4, and 5). When small amounts of protein were analyzed, two distinct bands corresponding to the glycosylated and deglycosylated forms were clearly visible in western blots (Fig. 3, lane 5). This antiserum did not react with P7.7, the peroxidase isozyme of M_r 65,000, or with other IWF proteins.

The amino acid content of deglycosylated P8.5 is given in Table II. The content of cysteine (estimated eight residues per polypeptide) and of histidine (estimated four residues per polypeptide) were similar to those found in other peroxidases. The amino acid composition of P8.5 is distinct from other cationic barley and wheat peroxidases that have been reported in the literature (Table II).

Table I. Yields and Recovery of P8.5 after Various Purification Steps

Purification Step	Protein mg	A ^a
Acetone-precipitated IWF	56.1	1.00
0.02 M sodium acetate eluate from CMC column	22.8	0.41
Rotofor separation, basic fraction	4.7	0.08
Gel filtration, peak 2	2.9	0.05

^a A, the proportion of protein coeluting with P8.5.

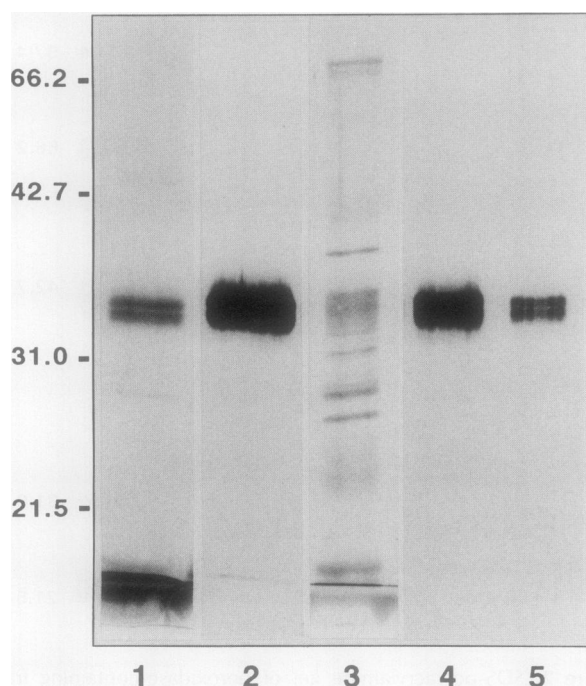


Figure 3. SDS-polyacrylamide gel and immunoblot of IWF and purified P8.5. Protein was resolved by SDS-PAGE and visualized by either silver staining (lanes 1 and 3) or by immunoblotting with an antiserum raised to deglycosylated P8.5 (lanes 2, 4, and 5). Lanes 1 and 2 contain 0.5 μg of purified P8.5; lanes 3, 4, and 5 contain IWF. To better visualize P8.5a and P8.5b, 10% as much sample was analyzed in lane 5 (0.37 μg of protein) as in lanes 3 and 4 (3.7 μg of protein/lane). The M_r ($\times 10^{-3}$) of reference standards are given to the left of lane 1.

DISCUSSION

Two extracellular barley leaf peroxidases increase in specific activity in response to infection by the obligate fungal pathogen, *E. g. hordei* (11). One peroxidase, P5.2, increases 4-fold, and the other, P8.5, increases 3-fold within 72 h of inoculation with this pathogen. As a first step in determining its *in vivo* role in host/pathogen interactions, P8.5 was purified.

A characteristic feature of peroxidases is the occurrence of eight cysteine residues and at least two histidine residues (17, 18). It has been proposed that four intramolecular disulfide bonds stabilize the three-dimensional configuration of peroxidases. The two histidine residues occur in highly conserved regions, the postulated heme-binding and catalytic sites. P8.5 has eight cysteine and four histidine residues and, thus, is similar to other peroxidases in this feature. The relative abundance of amino acids in P8.5 falls within the range observed for other cationic plant peroxidases (Table II). Once the amino acid sequence of P8.5 is known, more precise sequence comparisons will be possible.

Deglycosylation of a mixture of the two polypeptides of M_r 35,000 and 33,900 resulted in a single silver-stained band with a M_r of 33,900. Thus, the carbohydrate component of P8.5 represents 3% of the total M_r of this protein. The

contribution of carbohydrates to the M_r of other peroxidases is quite variable. Aibara et al. (1) observed that the M_r difference among six different peroxidases from horseradish was primarily due to the carbohydrate moiety. The carbohydrate moiety represented from 0.8 to 14.1% of the various horseradish peroxidase isoforms. Hu and van Huystee (9) commented that the carbohydrate moiety of an excreted, cationic peroxidase found in peanut suspension cultures represented 22% of its M_r . By contrast, a number of peroxidases have been isolated with no glycosyl groups (13, 25). The function of carbohydrates associated with peroxidases is unknown. Hu and van Huystee (9) demonstrated that enzyme activity and extracellular targeting was not affected by the glycosylation pattern. However, the polypeptide component was significantly more susceptible to proteolytic degradation *in vitro* in the absence of the carbohydrate moiety (9).

It is not clear why a subset of P8.5 is not glycosylated; however, a similar situation is found in other plants (28). In petunia, three isoforms of peroxidase *a* occur with M_r values of 37,500, 37,000, and 36,500. The observed M_r differences were attributed to the modification of peroxidase *a*1 the M_r 37,500 isoform, by an extracellular α -mannosidase (8). In barley endosperm, Rasmussen et al. (21) found two peroxidase isoforms, designated BP 1a (M_r 37,000) and BP 1b (M_r 36,000). Glucosamine analysis indicated that the M_r difference between BP 1a and BP 1b was due to the carbohydrate moiety. A similar mechanism may contribute to the observed M_r heterogeneity of P8.5. We propose to call the glycosylated P8.5 isoform P8.5a, and the isoform of M_r 33,900 that is lacking carbohydrate side chains P8.5b.

After gel filtration, the pooled fractions containing P8.5 had an R_z value of about 1.5. This value is relatively low when compared with some purified plant peroxidases; horseradish peroxidase isozymes with R_z values of 3.4 have been reported (1). However, van Huystee et al. (29) have documented changes in R_z values of purified peroxidases with storage. After 10 d of storage in 7% isopropanol at 4°C, a drop in R_z value from 2.5 to 0.8 was observed. In our study, ion-exchange chromatography and gel filtration were performed at room temperature. In addition, fractions collected during the purification procedure were stored for long periods of time at -20°C. Either factor could account for the relatively low R_z value of P8.5 in the current study.

Recently, Rebmann et al. (22) reported the sequence of a cDNA for a putative peroxidase whose mRNA accumulated in wheat leaves about 12 h after inoculation with the barley pathogen *E. g. hordei*. The deduced polypeptide had a predicted pI of 5.7 and may be homologous to the barley infection-related peroxidase isozyme P5.2.

Rasmussen et al. (21) have identified two peroxidase isoforms in the endosperm of barley that have some similarity to P8.5a and P8.5b. Although the pI values of these endosperm isozymes are similar to P8.5, their M_r values are slightly higher. In addition, mRNA coding for BP 1 was not detected in barley leaves, suggesting that the endosperm BP 1 peroxidase and P8.5 are distinct isozymes. Finally, based on a cDNA sequence of 158 amino acids of the carboxy terminus, BP 1 contains 16 arginine and 2 tyrosine residues (21). P8.5 contains only 1 tyrosine and 12 arginine residues. Thus, P8.5 and BP 1 are distinct peroxidases. A peroxidase isozyme of

Table II. Amino Acid Content of P8.5, a Wheat Infection-Related Peroxidase, and Several Cationic Peroxidases

Amino Acid	P8.5	Peroxidase ^a						
		A ^b	B	C ^b	D	E	F ^b	G ^b
		mole %						
Gly	10.0	7.24	8.9	7.61	8.11	5.26	7.14	9.12
Ala	9.2	11.38	12.2	11.42	10.81	7.12	9.86	8.79
Val	7.3	4.83	6.7	5.88	6.42	6.50	5.78	7.82
Leu	7.7	8.62	9.9	9.69	7.09	11.46	8.84	8.14
Ile	3.4	5.17	3.8	3.46	5.07	4.02	4.08	4.23
Ser	11.3	10.34	7.6	11.42	14.19	8.36	11.22	9.45
Thr	9.2	8.97	7.1	9.00	5.41	7.74	8.84	7.17
Cys	2.7	2.76	— ^c	2.77	2.70	2.48	3.06	2.61
Met	1.1	2.41	1.1	2.08	2.03	1.86	1.02	0.98
Asx	11.0	—	11.8	—	—	—	—	—
Asp	—	6.90	—	5.19	5.41	7.12	5.44	6.19
Asn	—	7.24	—	7.96	7.77	8.36	8.84	5.21
Glx	8.1	—	7.7	—	—	—	—	—
Glu	—	1.72	—	1.38	1.35	2.48	2.04	2.28
Gln	—	3.79	—	3.81	3.38	4.02	3.40	4.89
Arg	4.0	5.17	5.1	4.15	5.74	6.50	3.74	6.19
Lys	4.2	2.41	2.3	3.46	3.38	1.86	4.42	3.91
His	1.4	1.38	1.8	1.38	1.01	1.24	1.36	1.63
Phe	3.3	4.48	6.7	4.15	4.73	6.50	5.10	5.86
Tyr	1.0	1.38	1.7	1.38	1.35	1.55	2.04	1.30
Trp	—	0.34	—	0.35	0.34	0.31	0.34	0.65
Pro	5.1	3.45	5.5	3.46	3.72	5.26	3.40	3.58

^a A, Wheat infection-related peroxidase (GenProt Accession: WHTPERO) (1, 22); B, P5, barley leaf peroxidase with pI of 8.5 (24); C, cationic wheat peroxidase (GenProt Accession: WHTPEROXIG) (1); D, cationic turnip peroxidase, TP7 (Swiss Protein Accession: P00434) (17); E, cationic horseradish peroxidase C1 (Swiss Protein Accession: P00433) (17); F, cationic peanut peroxidase (GenProt Accession: ARCPNC1) (1); G, cationic peanut peroxidase (GenProt Accession: ARCPNC2) (1). ^b The site of signal peptide cleavage was estimated by the authors. The first amino acid of the mature polypeptide was not determined by N-terminal sequencing. ^c —, Not determined.

pI 8.5 was also purified from barley leaf tissue (24). The peroxidase from crude leaf extracts had a M_r of 44,000, was 15% carbohydrate, and had a different amino acid composition than P8.5 (Table II, ref. 24). Therefore, this isozyme appears to be different from the extracellular, infection-related P8.5 isozyme described here.

The diversity of peroxidase isozymes documented in barley serves to highlight the large number of peroxidases found in plants. Specific biochemical functions or physiological roles have not been assigned to most peroxidase isozymes that have been purified from plants to date (4, 28). The purification to homogeneity of P8.5 and the production of an anti-P8.5 antiserum represent a first step toward investigating the role of this peroxidase in host-pathogen interactions.

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