THE ISOLATION AND CHARACTERIZATION OF PEROXIDASE ISOZYMES IN WHEAT CARRYING THE *Sr6* ALLELES FOR RESISTANCE TO *PUCCINIA GRAMINIS* VAR. *TRITICI*

by

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ABBREVIATIONS

CM-cellulose : carboxymethylcellulose

DCP : 2,4-dichlorophenol

DEAE-cellulose: diethylaminoethylcellulose

DMAC : p-N,N'-dimethylaminocinnamaldehyde

EDTA : ethylenediaminetetraacetic acid

IAA : indoleacetic acid

PDA : p-phenylenediamine

TEMED : N,N,N',N'-tetramethylethylenediamine

Tris : Tris(hydroxymethyl)aminomethane

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INTRODUCTION

Peroxidases occur widely in higher plants and have been found to catalyze a variety of reactions in vitro. Some of the possibly biologically important ones are tryptophan oxidation (33, 57), IAA oxidation (15, 18, 25, 26, 44, 57), α-oxidation of fatty acids (42, 57), aromatic hydroxylation (6, 43, 57), oxidation of dihydroxyfumaric acid to α-ketosuccinate (7, 51, 57, 74), lignin formation (20, 21, 67, 68, 70, 71), and ethylene formation (17, 34, 41, 80).

In 1941, Theorell obtained the first crystalline samples of peroxidase from horseradish roots, and later suggested that more than one peroxidase may be present in a given source of enzyme. Subsequently, several other investigators found multiple components in peroxidase systems from other plant sources. The presence of multiple forms, or isozymes, could possibly explain the variety of reactions that are catalyzed by this enzyme.

Several investigators have examined the physico-chemical and catalytic properties of various peroxidase isozymes (28, 32, 47, 48, 56, 60, 63, 64, 65, 66, 77, 78). The structures of the horseradish peroxidase isozymes have been investigated by Shannon and co-workers (32, 63, 64), who suggested that they can be classified into three groups

depending upon their physico-chemical properties, and by their activities with certain hydrogen donors (O-dianisidine and oxalacetate). They obtained evidence to show that isozymes in a group have very similar primary structures, which differed from that of isozymes in the other groups. Differences in physico-chemical properties have also been detected among peroxidase isozymes from wheat (60, 65, 66), rice (28), Alaska pea (56), and Japanese radish (46, 47, 48). Despite the efforts so far expended, the precise physiological functions of peroxidase are not clear, especially since most of the assay systems used to study peroxidase isozymes employ substrates that do not appear to have biological importance.

A significant role has been attributed to peroxidases in plant disease reactions. Increase in peroxidase activity has been shown to be one of the most widespread biochemical symptoms of diseased, as well as injured, tissues (14, 27, 30, 49, 58, 59). The increases have been attributed to several factors, among which are certain compounds that increase during injury or disease such as ethylene (22, 23, 45, 53, 72) and certain amides and amino acids (14). Peroxidase increases have been suggested as being responsible for disease resistance by indirectly inhibiting pathogen growth through participation in the biosynthesis of phenolic compounds (6, 52), or by direct inhibition of fungal growth (40).

Seevers, et al. (58, 59) examined the possible role of peroxidases in resistance of wheat carrying the Sr6 alleles for resistance or susceptibility to Puccinia graminis var. tritici race 56. Using spectrophotometric assays, with p-phenylenediamine as substrate, they found that total peroxidase activity paralleled that observed for IAA oxidase activity (58), which, in turn, appeared to be correlated with the development of resistance to the pathogen (2). It was then suggested that the IAA decarboxylation lesion associated with resistance is an expression of increased peroxidase activity at the time when resistance is first detectable (8, 59).

When extracts from both resistant and susceptible tissues were subjected to disc electrophoresis using polyacrylamide gels and benzidine as substrate (59), 14 peroxidase isozymes were identified. Of these, only one identified as isozyme 9, increased consistently and significantly with infection of resistant lines. The increase was not as pronounced in susceptible lines. From the evidence obtained, isozyme 9 appeared to be mainly responsible for the observed changes induced in resistant reactions (59). However, other evidence also suggests that neither total peroxidase activity nor isozyme 9 are causally related to resistance. Rather, peroxidase increases apparently result from a non-specific incompatibility caused by a prior biochemical event (8, 11, 58, 59).

These observations suggested that rust-affected wheat leaves might be a useful system for elucidating some of the properties and biological functions of individual peroxidase isozymes, especially isozyme 9. Most of the wheat isozymes are synthesized de novo in the early stages of germination (38, 60). They are apparently of importance in the processes of growth and development, but none can be associated directly with a specific process in contrast to the association of isozyme 9 with resistance to rust.

Much of the previous work on the characterization of enzymatic properties of isozymes has been done using artificial substrates involving reactions of unknown or dubious physiological significance. Although some of the commonly used substrates provide means for relating the catalytic properties of wheat isozymes to those of other systems, a primary goal of this research was an attempt to determine if the individual isozymes varied in activity with substrates of possible significance to healthy or infected wheat leaves. IAA oxidation, ethylene production and lignin formation are common processes during growth and development, but also have been implicated as important aspects of disease resistance. They were chosen, therefore, for particular emphasis in this study after techniques for the purification of several wheat isozymes were developed.

Finally, the marked increase in activity of isozyme 9 offered an opportunity to establish whether the increases were due to de novo synthesis or to enzymatic activation. There are numerous reports of increases in enzyme activity during plant infection and they are generally assumed to be the result of protein synthesis although clear evidence for synthesis has not been presented.

MATERIALS AND METHODS

Growth and Inoculation of Plants

The experimental lines of wheat (*Triticum aestivum*) containing the *Sr6* alleles for resistance to race 56 of *Puccinia graminis* var. *tritici* were used for these experiments. Approximately 50 seeds were planted per pot, using 6 in. clay pots filled with autoclaved soil. The pots were placed in a growth room at 20 ± 1 C, under fluorescent lights (3000 ft. c.) with a 12-hour photoperiod.

When the primary leaves were fully developed, usually 7 to 8 days after planting, pots were randomly taken for inoculation. The uredospore inoculum was propagated on the susceptible variety Little Club, and was usually collected within a week from inoculation of experimental plants in order to ensure high viability. Approximately 5 to 10 mg uredospores per pot were used to give moderate to heavy infection.

Towards the end of the photoperiod, the pots were placed in a dew chamber (21 ± 1 C) and dusted with a mixture of uredospores and talc (approximately 50-100 mg spores per 2 g talc). The plants remained in the chamber for at least 15 hours and returned to the 20 C growth room. Control (uninoculated) plants, when required, remained in the growth room.

The inoculated resistant line, Sr6, was used for isolation and characterization of different peroxidase isozymes. For the D_2O-density gradient experiments, which are described in a later section, both resistant, Sr6, and susceptible, sr6, lines grown on Vermiculite were used.

Approximately 6 to 8 days after infection, when flecks are fully visible and sporulation had begun, the primary leaves were harvested, placed in moistened paper towels and brought to the laboratory. The leaves were weighed and sliced into approximately 1 cm sections.

Extraction of Tissue for Peroxidases

The extraction medium was 0.1 M Tris buffer, pH 8, containing 0.1% ascorbic acid, 0.1% cysteine-HCl, and 17% sucrose (73). Five mls of this solution were used per g of tissue. The tissue was blended in the cold using a Waring blendor for 1 min. at high speed. Solid residue was removed by squeezing through cheesecloth, followed by centrifugation at 20,000 x g at 2 C for 20 min. The supernatant liquid will be referred to as the crude extract.

Protein Determination

The assay procedure used to determine protein concentrations was based on the method of Lowry, et al. (36) with bovine serum albumin as the standard. To 0.3 ml of sample, containing between 10 to 40 ug protein per ml, 0.7 ml of Folin-Ciocalteu reagent (brought to 1 N) was added. After

mixing, the solution was allowed to stand for 15 min. Three mls of a solution containing 2% Na_2CO_3, 0.4% NaOH and 0.02% NaK tartrate (w/v) was added and mixed. After 30 min., the absorbance was measured at 750 nm, using a Beckman DB spectrophotometer.

Carbohydrate Determination

Five tenths ml of anthrone solution (0.2% in 95 ml conc. H_2SO_4 and 5 ml H_2O) was added to 0.2 ml of sample or glucose standard, mixed, and placed in a boiling water bath for 15 min. The solutions were read at 625 nm after cooling.

Disc Gel Electrophoresis

The method was based on Ornstein's and Davis' (50). Standard 7% polyacrylamide gels were prepared in 10 cm tubes (7mm diameter) using a gel solution consisting of the following:

3.0 ml gel buffer (3M Tris-HCl, pH 9.5 with 0.46% (v/v) TEMED)

4.5 ml acrylamide solution (56.0 g acrylamide plus 2.8 g N,N' methylene bisacrylamide to 150 ml with H_2O)

4.8 ml 30% (w/v) sucrose

3.0 ml 0.14% (w/v) ammonium persulfate

5.7 ml H_2O.

The gels were allowed to polymerize for at least 30 min. before use. Between 25 to 100 ul of sample were applied

onto the gels. For crude extracts, a 1:10 to 1:20 dilution was sufficient to detect activity. Electrophoresis was conducted routinely at 3 ma per tube for 1-1/3 hr. or 2-1/2 hr., using Tris-glycine buffer (6 g Tris plus 28.8 g glycine to 1 liter with H_2O, pH 8.3), diluted 1:10 with water, as the electrolytic buffer. After electrophoresis, the gels were incubated in a reaction mixture consisting of 10 ml 0.025% (w/v) benzidine in 0.2 M acetate buffer, pH 5.0, and 0.02 ml 1.5% (v/v) H_2O_2) for peroxidase assay. The gels remained in the reaction mixture for 4 hrs., after which they were rinsed with distilled water and stored in 0.2 M acetate buffer, pH 5.0, until scanned. Scanning was done at 340 nm, using a Gilford spectrophotometer equipped with a linear transport system, at a scan speed of 1 cm/min.

Isolation of Peroxidase Isozymes

Ammonium sulfate precipitation. The crude extract was brought to 50% saturation with ammonium sulfate (Baker reagent grade) at 0 C and a precipitate allowed to form for approximately 8 hrs. or overnight. The precipitate was separated by centrifugation at 20,000 x g for 20 min., and the supernatant liquid was brought to 95% saturation with ammonium sulfate. The solution was allowed to stand for approximately 8 hrs. or overnight at 0 C. Centrifugation at 20,000 x g for 20 min. followed, after which the precipitate was dissolved in, and dialyzed twice against, 0.01 M

phosphate buffer, pH 7.0. The 50-95% ammonium sulfate fraction was then subjected to column chromatography. All succeeding steps were conducted in the cold (2 C).

CM-cellulose chromatography. Starting with this and on each succeeding step in the isolation procedure, enzyme assays, mainly using p-phenylenediamine as substrate, and protein determinations were made on all fractions. Individual peroxidase isozymes were identified by disc gel electrophoresis.

The 50-95% ammonium sulfate fraction was taken and passed through a CM-cellulose column (5 cm x 76 cm) equilibrated in 0.01 M phosphate buffer, pH 7.0. The fractions were assayed for the isozymes and appropriate fractions were pooled, concentrated by the Diaflo (Aminco) procedure, and dialyzed twice against 0.005 M Tris-HCl buffer, pH 8.0.

DEAE-cellulose chromatography. An aliquot of pooled fractions from CM-cellulose chromatography was passed through a DEAE-cellulose column (2.5 cm x 63.5 cm) equilibrated in 0.005 M Tris-HCl buffer, pH 8.0, using the same buffer as the initial eluant. After the first group of isozymes were eluted out of the column, salt gradients of 0-0.1 M NaCl and 0.1 M-0.25 M NaCl in the same buffer, were applied to the column. Appropriate fractions were pooled and concentrated.

Sephadex G-200 chromatography. The isozymes were separated further by passing through a Sephadex G-200

column (2 cm x 150 cm) using H_2O as the eluant.

To obtain pure isozymes, a second and sometimes a third passage through Sephadex G-200 column was necessary, applying only pooled fractions rich in the desired component.

Enzyme Assays.

A. Spectrophotometric assays for peroxidase. The following reaction mixtures were used.

p-phenylenediamine: λ = 485 nm

0.1 ml p-phenylenediamine (PDA), 1% aq.
0.1 ml H_2O_2 0.1%
1.9 ml sodium phosphate buffer, 0.1 M
0.1 ml enzyme

Guaiacol: λ = 470 nm

0.4 ml guaiacol, .02 M
0.1 ml H_2O_2, 0.1%
1.6 ml sodium phosphate buffer, 0.1 M
0.1 ml enzyme

Eugenol: λ = 425 nm

0.2 ml eugenol, 0.025 M, in 50% MeOH
0.1 ml H_2O_2, 0.1%
1.8 ml sodium phosphate buffer, 0.1 M
0.1 ml enzyme

o-dianisidine: λ = 470 nm

0.05 ml o-dianisidine, 1% in abs. MeOH

0.1 ml H_2O_2, 0.1%

1.95 ml sodium phosphate buffer, 0.1 M

0.1 ml enzyme

B. IAA oxidase assays.

Decarboxylation assay. The usual reaction mixture consisted of the following:

0.1 ml 30 mM $MnCl_2$

0.1 ml 1.5 mM 2,4-dichlorophenol (DCP)

0.5 ml IAA- $^{14}COO^-$ K^+, 500 ug/ml

1.8 ml 0.1 M NaH_2PO_4 (pH 4.7)

0.5 ml enzyme or extract.

The solutions were added in sequence into glass vessels (2 in. (dia.) x 4 in.) and covered with a rubber stopper bearing a planchet holder into which a glass planchet containing 0.1 ml 10% KOH was placed. The glass vessels were covered with black electrical tape for reactions carried out in the dark. The reaction was carried out at 30 C in a water bath with constant shaking. Planchets were replaced at different time intervals, and counted in a gas-flow counter (Tracer-Lab).

Gel assays. Gels were prepared and undiluted extracts subjected to electrophoresis as described earlier. Approximately 5 times more enzyme was required, relative to the

peroxidase gel assay. Two methods were used:

a. Endo's method (Fast Blue BB dye) (15).

After the electrophoretic run, the gels were placed in a reaction mixture consisting of the following:

0.5 ml 30 mM $MnCl_2$

0.5 ml 1.5 mM DCP

6.0 ml buffer (0.2 M phosphate, pH 4.7 or 0.2 M acetate, pH 5.0)

2.0 ml IAA, K-salt, 640 ug/ml

1.0 ml Fast Blue BB dye (Sigma)

Incubation was carried out for 8 hrs. in the dark, after which the gels were washed with distilled water, stored in buffer, and scanned as described previously.

b. Dimethylaminocinnamaldehyde (DMAC) method (19, 44).

After electrophoresis, the gels were incubated overnight in the reaction mixture similar to that in Endo's method, except that Fast Blue BB dye was not added. The isozymes were visualized by transferring the gels at the end of the incubation period to a 0.5% p-N,N' dimethylaminocinnamaldehyde solution in 1 N HCl. After 30 min. to 1 hr. of incubation, the gels were transferred to 1 N HCl, and scanned immediately at 562 nm.

C. Ethylene formation.

The method of Yang (80) was used. The reaction mixture consisted of the following:

1.0 ml 0.1 M phosphate buffer, pH 7.8

0.1 ml 20 mM resorcinol

0.1 ml 1 mM EDTA

0.1 ml 10 mM Methional

0.1 ml enzyme

0.1 ml $NaHSO_3$

to 2 ml with H_2O

The components were pipeted into 25 ml erlenmeyer flasks, the exact volumes of which have been previously measured, with $NaHSO_3$ pipeted last. The flasks were immediately sealed with rubber syringe caps, and incubated at 30 C with shaking. Two-ml samples were taken at time intervals and injected into the gas chromatograph (Hewlitt-Packard). Ethylene formation was determined by tracing the peaks on No. 16 bond paper, and weighing the tracings. A standard curve for ethylene was prepared similarly.

Isoelectric Focusing

A 0-50% sucrose gradient, containing 1% ampholine (LKB), was prepared in a 30 ml plastic tube by layering 5 ml each of 0, 10, 20, 30, 40 and 50% sucrose solutions (in 1% ampholine) with the sample in the 20% layer. The sucrose gradient was allowed to develop overnight. The tube then was punctured at the bottom with a No. 17 needle and pumped at 1 ml/min. into the isoelectric focusing apparatus (ISCO) using 60% sucrose and an ISCO pump. The

lower electrolytic chamber (anode) was filled with 5% H_3PO_4 in 40% sucrose, and the upper electrolytic chamber (cathode), with 5% ethanolamine. Electrophoresis was carried out at constant voltage, usually 800 v, for at least 36 hrs. with pH 3-10 ampholine, and 60 hrs. with narrower pH ranges.

After electrophoresis, the gradient was pumped out at 0.5 ml/min with the aid of 60% sucrose and 0.5 ml fractions collected. Enzyme activity and pH were assayed in alternate fractions. The pH was measured by diluting the fractions 1:10 with water. A 0.02-0.03 pH change was observed in doing this; however, it facilitated pH measurements at higher sucrose concentrations.

Studies on Protein Synthesis

A. $^{14}CO_2$-labeling. Eight pots of Sr6 were planted and inoculated with wheat stem rust uredospores as described earlier. A day after inoculation, the pots were placed in an enclosed chamber into which 200 uCi of $^{14}CO_2$ was generated using NaH $^{14}CO_3$ (0.825 mg) and cold $NaHCO_3$ (0.9 mg) with 5 ml 6 N H_2SO_4. This was followed by daily treatments of 100 uCi $^{14}CO_2$ for 6 days. The primary leaves were harvested and the peroxidases were extracted as described previously. The extract was divided into several portions and frozen until ready for use.

The extracts were subjected to the isolation procedures described to obtain purified isozymes. Aliquots were

taken and assayed for protein, radioactivity and peroxidase activity during each isolation and purification step.

B. D_2O labeling - Density Gradient Centrifugation Experiments.

1. Isolation of Isozymes. Plants used for this experiment were grown in Vermiculite (Terra-Lite brand). The Vermiculite was washed with water before use. Both Sr6 and sr6 were planted in plastic pots (5" x 5" x 3") at a rate of 100-120 seeds per pot. The plants were supplied with water only until the seeds had begun to germinate (or the growing plant became visible), which usually occurs 2 days after planting. They were then watered daily with a modified Hoagland nutrient solution (36). Every 6th or 7th day, the plants were supplied only with water to prevent build-up of nutrients. When the primary leaves were fully developed, 2 pots each of Sr6 and sr6 were taken and inoculated. A day after inoculation, one half of the pots (uninoculated Sr6/sr6, inoculated Sr6 and inoculated sr6) were watered with 150 ml of 40% D_2O and the other half with water. This was done daily up to the 6th day after inoculation (5 days of D_2O-treatment). On day 7, the primary leaves were harvested, and peroxidases extracted. Isozymes 9 and 10 were primarily the isozymes of interest and the isolation procedures were fashioned to obtain these 2 isozymes.