Bactericidal Activity of Horseradish Peroxidase on *Xanthomonas phaseoli* var. *sojensis*

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ABSTRACT

Horseradish peroxidase (HRP) was bactericidal on *Xanthomonas phaseoli* var. *sojensis,* a bacterium pathogenic on soybeans, in the presence of potassium iodide (KI) and hydrogen peroxide (H_2O_2). The antibacterial activity was greatest when HRP was used at 50 μg/ml. Concns of HRP greater than 50 μg/ml did not increase the bactericidal activity. The antibacterial activity of HRP was largely prevented either by preheating the enzyme at 80 C for 20 min or by deleting H_2O_2 in the assay system. The addition of ascorbic acid (20 nmoles/ml) to the assay system, increased the bactericidal activity of HRP. Substituting dehydroascorbic acid for ascorbic acid further enhanced the bactericidal activity of HRP, resulting in total loss in viability of the bacterium.

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Additional key words: bacterial pustule, enzyme.

Peroxidase is a heme protein that is widely distributed in higher plants, animals, and microorganisms (2, 18, 20). However, its concn generally is higher in plants than in animals. Peroxidase in animals is believed to function in phagocytosis. This enzyme in combination with hydrogen-peroxide and a halide is virucidal (1), mycoplasmacidal (8), bactericidal (10), and fungicidal (11). Peroxidase activity increases in both resistant and susceptible plants in response to infection by viruses (4, 5, 12, 14), fungi (6, 9, 17, 19), and bacteria (13). But the function of the additional

peroxidase, if any, has not been established. It therefore seemed of interest to determine whether plant-produced peroxidase would have any antimicrobial activity on plant pathogens. This study was initiated to develop an assay system to determine the bactericidal effect of horseradish peroxidase (HRP) on plant pathogenic bacteria.

MATERIALS AND METHODS.—*Bacteria.*—*Xanthomonas phaseoli* (Smith) Dawson var. *sojensis* (Hedges) Starr and Burkholder, the inciting agent of bacterial pustule disease of soybeans was used as the test organism.

Buffers.—Buffers used were the same as those described by Gomori (7).

Assay of bactericidal activity.—*X. phaseoli* var. *sojensis* cells were prepared for bacterial assay as follows: Bacterial cells were incubated for 16 to 18 h at 30 C in trypticase soybroth (TSB). A 1-ml aliquot from this culture was transferred into 0.25% TSB and incubated at 30 C in a rotary shaker. Growth was monitored by measuring the change in optical density (OD) at 540 nm. When OD level reached 0.34, the cells were washed three times in citrate phosphate buffer (pH 5.0) and suspended in 3 ml of the same buffer. This suspension was adjusted to OD 0.75 and diluted 10-fold to obtain 10^6 cells/ml. The bactericidal assay procedure was the same as that of Lehrer (11), except that the source of enzyme and the concn of components were modified. The assay components, in a final volume of 2 ml, were placed in sterile tubes (12 × 95 mm) in a temp-controlled shaker incubator, incubated for 30 min at 37 C while being shaken at 100 cycles/min. Unless otherwise noted, the components were added in the following amounts: 0.33 ml of citrate phosphate buffer (pH 4.1); 0.42 ml of potassium iodide (10 μmole); 0.14 ml of hydrogen peroxide (3×10^{-3} M); 0.25 ml of horseradish peroxidase (400 μg/ml) obtained from Nutritional Biochemicals (RZ = 1); 0.5 ml of bacterial cells (10^6 cells/ml); and 0.36 ml of sterile distilled water. After incubation, triplicate samples were diluted with TSB, spread on trypticase soy agar plates, and further incubated for 24-48 h before the bacterial colonies were counted. The bactericidal activity of the peroxidase was assessed by comparing the bacterial concns in the test reaction mixtures with the concns found in the control tubes containing only the test organism, buffer, and distilled water. The following formula was used to express percentage of survival.

$$\text{Percent survival} = \frac{\text{number of bacterial cells in treatment tubes}}{\text{number of bacterial cells in buffer control tubes}} \times 100$$

Because growth or death of bacteria is expressed logarithmically, percentage of survival was plotted on log scale, and a difference of 1 log unit between treatments was considered significant.

Determination of enzyme activity.—Peroxidase activity was assayed by adapting the procedure of Luck (15). Enzyme assay was based on the oxidation of *p*-phenylenediamine in presence of hydrogen peroxide and quantified spectrophotometrically by using a Bausch and Lomb Spectronic spectrophotometer at 485 nm. The reaction medium consisted of 1.0 ml *p*-phenylenediamine (1.0%); 0.4 ml hydrogen peroxide (3×10^{-3} M); 1.0 ml citrate phosphate buffer (pH 4.1); 1.0 ml enzyme; and enough distilled water to bring total volumes to 6 ml. Changes in OD were recorded at 20-sec intervals. Peroxidase activity was expressed as units of activity/mg protein and was calculated by using the formula.

$$\frac{\frac{\triangle OD}{min} \times \text{dilution of the enzyme used for the assay}}{\text{mg protein}}$$

Changes in OD against time were plotted, and the first three points that lay on a straight line that cut the ordinate at zero time were used.

Determination of the effect of various concns of either ascorbic acid or dehydroascorbic acid on survival of X. phaseoli var. sojensis.—The assay mixture consisted of an appropriate volume of buffer, bacterial cells, and one concn of either ascorbic acid or dehydroascorbic acid. The total volume of the reaction mixture was 2 ml. The concn of ascorbic acid and dehydroascorbic acid used ranged from 20 nmoles/ml to 20 μmoles/ml. The incubation time was 30 min. Bactericidal effect was determined by taking viable cell counts of each treatment.

RESULTS.—*HRP assay system.*—Incubation of *X. phaseoli* var. *sojensis* in the presence of HRP, hydrogen peroxide (H_2O_2) and potassium iodide (KI) for 30 min resulted in only 0.09% survival of bacterial cells. The assay components, alone or in all other combinations, had no significant effect on the survival of bacterial cells (Table 1). Thus, all the components in the assay system are necessary for the peroxidase to be effective in its bactericidal activity. The heated HRP did not have any bactericidal activity when it was used with all the components of the assay system (Table 1).

Bactericidal activity of HRP on X. phaseoli var. sojensis in presence of ascorbic or dehydroascorbic

TABLE 1. The effect of horseradish peroxidase (HRP) on survival of *Xanthomonas phaseoli* var. *sojensis*

Treatment number	Component	Survival (%)
1	CPB[a]	100.00
2	CPB + H_2O_2	100.00
3	CPB + H_2O_2 + KI	67.00
4	CPB + KI + HRP[b]	100.00
5	CPB + KI	89.00
6	CPB + H_2O_2 + KI + HRP	0.09
7	CPB + H_2O_2 + KI + HRP (heated enzyme)	100.00

[a]Citrate phosphate buffer.
[b]Enzyme activity in units 3,625/mg protein/min.

acid.—The bactericidal activity of HRP (50 μg/ml) increased when ascorbic acid (20 nmoles/ml) was incorporated in the assay mixture. This increase resulted in 0.001% survival of bacterial cells (Table 2). When ascorbic acid was replaced with dehydroascorbic acid (20 nmoles/ml), the dehydroascorbic acid further increased the bactericidal activity of HRP, resulting in a total loss in viability of bacterial cells.

Determination of optimum concn of HRP on survival of X. phaseoli var. sojensis.—Preliminary studies indicated that HRP (50 μg/ml) was bactericidal in the assay system. We tested HRP at concns of 50, 100, 250, 500, and 1,000 μg/ml. Bactericidal activity was maximum at 50 μg/ml (Fig. 1).

Effect of incubation time on survival of X. phaseoli var. sojensis.—The effect of incubation time on the percentage of survival of bacterial cells was determined in the presence of HRP (50 μg/ml), with and without ascorbic acid (20 nmoles/ml). After the assay mixture was incubated for 15, 30, 45, or 60 min, the number of viable cells was determined by plating at each time interval of each treatment. There was a significant decrease in percentage survival of the bacterial cells at 15-min incubation time (Fig. 2). Furthermore, HRP with ascorbic acid had greater bactericidal activity than did HRP alone. When the incubation time was extended for 45 min, decrease in percentage of survival was significant in two treatments (HRP and HRP with ascorbic acid). There also were significant differences between the two treatments, at 30 min and 1 h (Fig. 2).

Effect of various concns of either ascorbic acid or dehydroascorbic on survival of Xanthomonas phaseoli var. sojensis.—The results to this point have demonstrated that ascorbic acid, under certain conditions, enhances the bactericidal effect of peroxidase. The experiments were performed to resolve whether ascorbic acid or dehydroascorbic acid alone had any bactericidal effect. Ascorbic acid at a concn of 20,000 nmoles/ml and 2,000 nmoles/ml had bactericidal effect on *X. phaseoli* var. *sojensis* resulting in 0.02% and 0.18% survival of bacterial

TABLE 2. The effect of horseradish peroxidase in combination with either ascorbic acid or dehydroascorbic acid on survival of *Xanthomonas phaseoli* var. *sojensis*

Treatment number	Component	Survival (%)
1	CPB [a]	100.000
2	CPB + ascorbic acid[b]	100.000
3	CPB + dehydroascorbic acid[c]	100.000
4	CPB + H_2O_2 + KI + HRP[d]	0.090
5	CPB + H_2O_2 + KI + HRP + ascorbic acid	0.001
6	CPB + H_2O_2 + KI + HRP + dehydroascorbic acid	0.000

[a]Citrate phosphate buffer.
[b]20 nmoles/ml.
[c]20 nmoles/ml.
[d]Enzyme activity in units 3,625/mg protein/min.

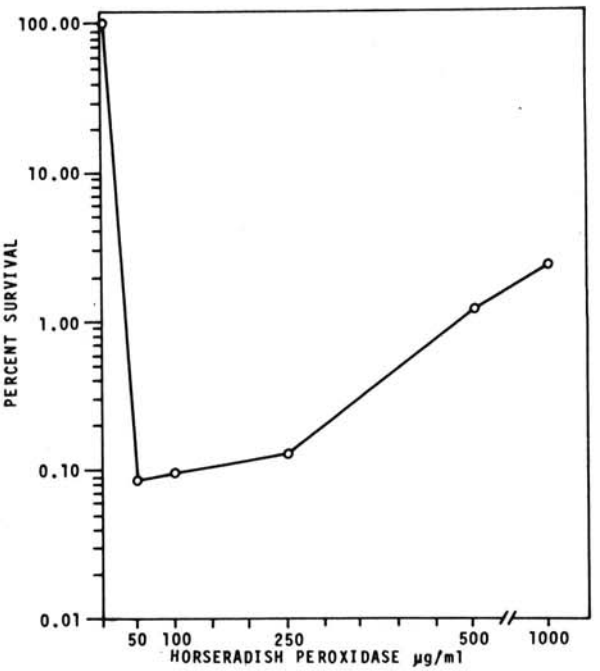

Fig. 1. Effect of various concns of horseradish peroxidase on survival of *Xanthomonas phaseoli* var. *sojensis.*

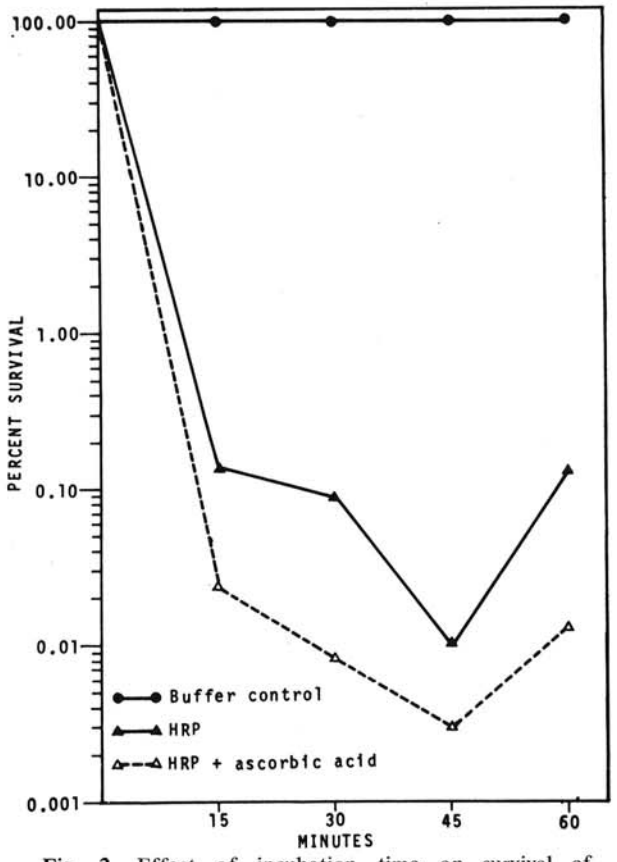

Fig. 2. Effect of incubation time on survival of *Xanthomonas phaseoli* var. *sojensis* in presence of horseradish peroxidase, with and without ascorbic acid.

TABLE 3. Effect of concn of either ascorbic acid or dehydroascorbic acid on survival of *Xanthomonas phaseoli* var. *sojensis*

Concn (nmoles/ml)	Survival	
	Ascorbic Acid (%)	Dehydroascorbic acid (%)
20	100.00	100.00
200	100.00	100.00
2,000	0.18	100.00
20,000	0.02	0.09

cells, respectively. Dehydroascorbic acid, however, was bactericidal at only a concn of 20,000 nmoles/ml, which resulted in 0.09% survival (Table 3).

DISCUSSION.—These data indicate that *X. phaseoli* var. *sojensis*, the soybean pathogen, is susceptible to a peroxidase-mediated antimicrobial system. This is the first report of the bactericidal activity of peroxidase on plant pathogenic bacteria. Earlier researchers (1, 11) have used HRP to study the effect of peroxidase on animal pathogens.

Another important aspect of the model presented, is that it provides a system in which many of the compounds oxidized by peroxidase could be screened for bactericidal effect. When ascorbic acid (20 nmoles/ml) was added to the assay components, the bactericidal activity of HRP increased. When ascorbic acid was replaced with dehydroascorbic acid (20 nmoles/ml) in the assay mixture, bactericidal activity of HRP further increased, resulting in total loss of viability of bacterial cells. Ascorbic acid or dehydroascorbic alone at the equivalent molar concn did not have bactericidal activity. Ericsson and Lundbeck (3), and Myrvik and Volk (16) also reported that oxidized products of ascorbic acid are bactericidal, but peroxidase was not involved in their assay system. When HRP was replaced with peroxidase isolated from soybean cultivars resistant ('Clark 63') and susceptible ('Clark') to bacterial pustule, we observed that soybean peroxidase also had bactericidal activity on *X. phaseoli* var. *sojensis* (Urs and Dunleavy, *unpublished*). This activity was greater in bacterial pustule-resistant cultivar Clark 63 than in the susceptible cultivar Clark.

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