# Inhibitory Effects of a Polyphenol-Polyphenol Oxidase System on the Infectivity of Cowpea Chlorotic Mottle Virus Ribonucleic Acid

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ABSTRACT

Solutions of enzymatically oxidized L-β-3, 4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, and catechol reduced the infectivity of cowpea chlorotic mottle virus ribonucleic acid (CCMV-RNA) as compared with the unoxidized phenolics, polyphenoloxidase only, and buffer controls. RNA infectivity decreased as the length of time the RNA was exposed to the oxidized phenolics increased from 0 to 60 min, or as the temperature of the reaction mixture increased from 0 C to 35 C. Oxidized chlorogenic acid reduced infectivity more effectively than either oxidized catechol or oxidized L-DOPA. Polyphenoloxidase (PPO) reduced the infectivity of CCMV-RNA by nearly 70% in 20 min at 23 C. Solutions of boiled PPO, bovine serum albumin (BSA), and boiled BSA, had little effect on RNA infectivity. Treatment of PPO with bentonite did not restore the infectivity of RNA to the level of the buffer control. It is suggested that the effects of oxidized phenolics on the infectivity of CCMV-RNA may be due to reactions between amino groups present on the RNA molecules and quinones.

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*Additional key words:* tyrosinase, RNase.

The hypersensitive necrotic reaction has been called the most important defense mechanism of plant tissues evoked by the attack of special strains of obligate parasites (4). When infected by certain viruses, many plants respond in a hypersensitive manner by forming necrotic local lesions which usually results in a localization of the infection. Quinones are thought to play an important role in the formation of local lesions (4, 15). These compounds inactivate a number of viruses in vitro (7, 10, 12, 13, 14), however the mechanism of inactivation is unclear. It has been suggested that quinones may inactivate viruses through interactions with the viral RNA (10, 11, 13, 14). It has been reported that tobacco mosaic virus RNA (TMV-RNA) is not affected by oxidized chlorogenic acid (9). Peanut stunt virus-RNA (PSV-RNA) is not affected by concentrations of tetrachlorobenzoquinone (TCQ) capable of inactivating intact PSV (13); however, southern bean mosaic virus RNA is completely inactivated by concentrations of TCQ having little effect on the intact virus (11).

This paper reports on the effects of three phenolics (and their oxidation products) normally found in plants, and polyphenoloxidase on the infectivity of ribonucleic acid isolated from cowpea chlorotic mottle virus.

MATERIALS AND METHODS.— Cowpea chlorotic mottle virus (CCMV) was maintained in cowpea, *Vigna sinensis* (Torner) Savi 'Early Ramshorn'. Purification of CCMV was carried out according to the method of Gay and Kuhn (6). CCMV-RNA was isolated using the method of Bancroft et al. (2) and stored at −20 C until used. Local lesion assays were made on the primary leaves of soybean, *Glycine max* L. 'Harosoy'.

Tyrosinase (polyphenoloxidase) (Sigma Chemical Company, St. Louis, Mo.) was dissolved in 0.01 M tris buffer pH 7.4 which contained 0.01 M KCl and 0.0001 M $MgCl_2$ (TKM buffer) (2) and used at a concentration of 0.2 mg/ml in all experiments. CCMV-RNA was thawed and diluted in TKM before each experiment. The final concentration of RNA, although constant for a given experiment, varied among experiments, averaging 3.2 μg/ml for the temperature experiments and 1.9 μg/ml for the time-course assays. L-β-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic-acid, and catechol were dissolved in TKM and used in all experiments at a final concentration of $1.25 \times 10^{-3}$ M. Temperature experiments were carried out for 15 min using temperatures of 0, 23, and 35 C. At zero time, 0.5 ml RNA, 0.25 ml polyphenoloxidase (PPO), and 0.25 ml of the appropriate phenolic solution were mixed in a glass vial (oxidized phenolic treatment). After 3 min, 0.5 ml RNA was added to 0.5 ml TKM in a second vial (buffer control). Three minutes later, 0.25 ml TKM, 0.25 ml phenolic solution, and 0.5 ml RNA were mixed in a third vial (unoxidized phenolic treatment). Nine minutes after the first vial was prepared, 0.5 ml RNA, 0.25 ml TKM, and 0.25 ml PPO were mixed in a fourth vial (PPO treatment). The preparation in each vial was inoculated on eight soybean half-leaves 15 min after being mixed.

TABLE 1. Local lesions produced by cowpea chlorotic mottle virus ribonucleic acid exposed to different compounds and to different temperatures for 15 min before inoculation [a]

| Treatment [b] | No. of local lesions/half-leaf | | |
|---|---|---|---|
| | 0 C | 23 C | 35 C |
| Buffer control | 138 [c] | 111 | 147 |
| Oxidized DOPA | 113 | 44 | 2 |
| Unoxidized DOPA | 162 | 106 | 68 |
| Oxidized chlorogenic acid | 88 | 1 | 0 |
| Unoxidized chlorogenic acid | 123 | 87 | 72 |
| Oxidized catechol | 115 | 24 | 11 |
| Unoxidized catechol | 120 | 35 | 6 |
| Polyphenoloxidase | 133 | 73 | 76 |

[a] Average concentration of RNA = 3.2 μg/ml.

[b] All phenolics used at a final concentration of $1.25 \times 10^{-3}$ M; final concentration of polyphenoloxidase = 0.05 mg/ml.

[c] Each figure represents the average of six replications; each replication consisted of inoculations on eight half-leaves of *Glycine max* 'Harosoy'.

Treatments in the time-course assays were prepared as described for the temperature experiments. Inoculations were conducted 0-1.5, 30, and 60 min after the solutions were mixed; however, oxidation was allowed to proceed for two min before inoculation in the zero time oxidized phenolic treatment, to allow for generation of the quinone.

Experiments were run to determine the nature of the inactivating effect of PPO on CCMV-RNA. Six treatments were compared. These were: (i) 0.5 ml RNA (0.69 $\mu$g/ml) + 0.5 ml TKM; (ii) 0.5 ml RNA + 0.25 ml PPO + 0.25 ml TKM; (iii) 0.5 ml RNA + 0.25 ml boiled (15 min) PPO + 0.25 ml TKM; (iv) 0.5 ml RNA + 0.25 ml bovine serum albumin (BSA) (0.2 mg/ml) + 0.25 ml TKM; (v) 0.5 ml RNA + 0.25 ml boiled (15 min) BSA + 0.25 ml TKM; and (vi) 0.5 ml RNA + 0.25 ml bentonite-treated PPO + 0.25 ml TKM. Inoculations of soybean leaves took place 20 min after the preparation of each vial. Bentonite-treated PPO was prepared by adding 1.0 ml bentonite, made according to Fraenkel-Conrat et al. (5), to 9.0 ml PPO, giving a concentration of PPO of 0.2 mg/ml. After mixing for 10 min at 0 C, the mixture was ultracentrifuged at 4 C for 1 hr at 135,000 *g* and the bentonite pellet was discarded. The experiment was repeated four times at 23 C.

RESULTS.— The results of the temperature experiments are shown in Table 1. In general, infectivity decreased with increasing temperature. The oxidized chlorogenic acid treatment always caused the greatest reduction in infectivity at any given temperature. All three oxidized phenolic treatments reduced infectivity nearly 100% at 35 C. With the exception of unoxidized catechol, the unoxidized phenolic treatments did not have such drastic effects on infectivity, although considerable reductions did occur compared to buffer controls. Of all phenolic treatments, unoxidized DOPA had the least, and oxidized chlorogenic acid the greatest, effect on infectivity. At 35 C polyphenoloxidase reduced the infectivity of CCMV-RNA nearly 50%.

The results of the time-course assays are shown in Table 2. With the exception of unoxidized chlorogenic acid, all treatments involving phenolics reduced the infectivity of CCMV-RNA nearly 100% in 60 min; however, the rate of reduction was much greater in the oxidized phenolic treatments. CCMV-RNA exposed to oxidized chlorogenic acid for the shortest time interval, showed a reduction in infectivity of nearly 75%. No lesions were detected when CCMV-RNA was exposed to oxidized chlorogenic acid at 30 or 60 min. Polyphenoloxidase alone reduced infectivity nearly 100% in 60 min, whereas the infectivity of RNA dissolved in TKM decreased nearly 50% in the same time period.

TABLE 2. Local lesions produced by cowpea chlorotic mottle virus ribonucleic acid exposed to different compounds for 0-1.5, 30, and 60 min at 23 C [a]

| Treatment [b] | No. of local lesions/half-leaf | | |
|---|---|---|---|
| | 0-1.5 min | 30 min | 60 min |
| Buffer control | 149 [c] | 111 | 78 |
| Oxidized DOPA | 107 | 4 | 0 |
| Unoxidized DOPA | 148 | 41 | 3 |
| Oxidized chlorogenic acid | 40 | 0 | 0 |
| Unoxidized chlorogenic acid | 102 | 89 | 66 |
| Oxidized catechol | 105 | 13 | 1 |
| Unoxidized catechol | 115 | 54 | 3 |
| Polyphenoloxidase | 142 | 36 | 0 |

[a] Average concentration of RNA = 1.9 $\mu$g/ml.

[b] All phenolics used at a final concentration of $1.25 \times 10^{-3}$ M; final concentration of polyphenoloxidase = 0.05 mg/ml.

[c] Each figure represents the average of four replications; each replication consisted of inoculations on eight half-leaves of *Glycine max* 'Harosoy'

The results of the experiments on the nature of the inactivating effect of polyphenoloxidase are shown in Fig. 1. As in previous experiments, active PPO had a marked effect on CCMV-RNA, causing a reduction in infectivity of nearly 70%. Solutions of BSA, boiled BSA, and boiled PPO had little effect on infectivity. Treatment of PPO with bentonite reduced the inactivating effect of PPO, but infectivity was still low as compared to the buffer control.

DISCUSSION.— Infectivity of CCMV-RNA was reduced not only by the oxidized phenolics, but also by the unoxidized phenolics, polyphenoloxidase, and bentonite-treated polyphenoloxidase. The infectivity of RNA dissolved in buffer also decreased with time. The latter reduction in infectivity may be due to an inherent instability of the RNA, or to traces of RNase present in the RNA preparation or in the buffer, or to traces of autoxidizable compounds; e.g., phenolics, present in the RNA preparation. The unoxidized phenolic compounds may have reduced infectivity by reacting directly with the RNA. Cheo and Lindner (3) suggested that tannic acid reduced the infectivity of TMV by reacting with TMV-RNA. An alternative explanation is that autoxidation of the phenolics occurred during exposure to the RNA, and the oxidized products, having greater reactivity than the reduced phenols (8), were responsible for the reduced infectivity.

The mechanism by which PPO reduced the infectivity of CCMV-RNA is not clear. One would not expect that RNA could be utilized as a substrate by the enzyme since nucleic acids are not closely related in structure to polyphenols. In a separate experiment designed to demonstrate whether or not PPO was contaminated with RNase, PPO and RNA were incubated together for 12 hr at 23 C. At the end of the incubation period the mixture was tested for the release of acid-soluble nucleotides which would have indicated presence of RNase in the PPO preparation. However, only traces of such nucleotides could be detected, indicating that the PPO preparation used was free of RNase. A non-specific attraction between RNA and the PPO protein as the reason for the inactivation is argued against by the fact that BSA at the same concentration as PPO had no effect on the infectivity. Treating the PPO with bentonite decreased somewhat the inactivating effect; inactivation was negligible when the RNA was exposed to boiled PPO. It is possible, therefore, that a heat-labile factor is present in the PPO which can inactivate RNA without causing hydrolysis. Another most interesting possibility would be the presence in the RNA preparation of a few molecules of polyphenols per RNA molecule which, whether in solution or loosely associated with the RNA, upon exposure to PPO or upon autoxidation would form quinones that could react with and inactivate the viral RNA.

The oxidized phenolic treatments have been shown to greatly reduce the infectivity of CCMV-RNA. While it

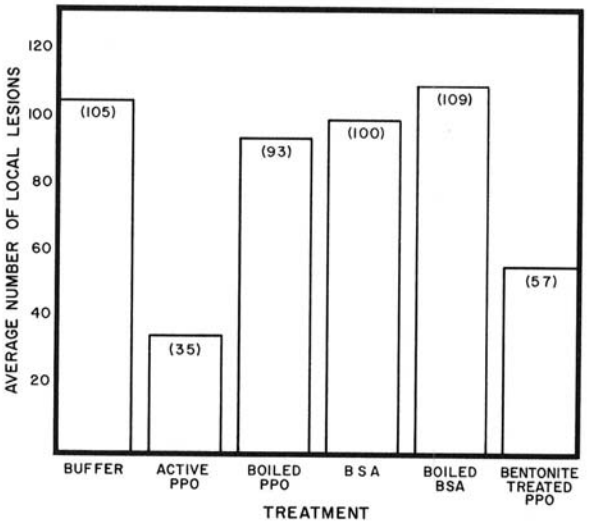


**Fig. 1.** Infectivity changes in CCMV-RNA exposed to polyphenoloxidase, boiled polyphenoloxidase, bovine serum albumin (BSA), boiled BSA, and bentonite-treated polyphenoloxidase for 20 min at 23 C. Final concentration of RNA = 0.69 $\mu$g/ml; final concentrations of BSA and PPO = 0.05 mg/ml.

is possible that some of the reduced infectivity may be due to effects of the oxidized compounds on the assay plant, and not on the RNA, we have reasons to believe otherwise. Other workers have shown that reductions in infectivity of whole viruses by oxidized phenolics have not been caused by effects of the oxidized compounds on the assay plant (7, 14). In our experiments, infectivity of CCMV-RNA exposed to the oxidized compounds decreased both with increasing time and temperature. A time effect as demonstrated herein would not be expected if reduction in infectivity were due only to effects of the treatments on assay host susceptibility. The results are much more readily explainable on the basis of reactions occurring between the RNA and the oxidized phenolics. A temperature effect as demonstrated is also inconsistent with the argument that host susceptibility is affected since only moments are required for the applied inoculum to reach the ambient temperature of the leaf surface.

Of the four nitrogenous bases present in CCMV-RNA (2), adenine, cytosine, and guanine possess free amino groups. Based on the great reactivity of quinones (1, 8) and the affinity of oxidized phenolics for amino groups (8), it is possible that the amino groups of the three aforementioned nitrogenous bases may be points of attachment of quinones to viral RNA, and that reactions such as these may play an important role in the inactivation of viruses by plants.

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