Plant Physiol. (1970) 46, 752–753

Short Communication

Inhibition of Ornithine Carbamyl Transferase from Bean Plants by the Toxin of *Pseudomonas phaseolicola*[1]

Received for publication July 9 1970

SURESH S. PATIL
Department of Plant Pathology, University of Hawaii, Honolulu, Hawaii 96811
P. E. KOLATTUKUDY
Department of Agricultural Chemistry, Washington State University, Pullman, Washington 99163
A. E. DIMOND
Department of Plant Pathology and Botany, The Connecticut Agricultural Experiment Station, P.O. Box 1106, New Haven, Connecticut 06504

Pseudomonas phaseolicola (Burkh.) Dowson, a pathogen of bean (*Phaseolus vulgaris* L.) plants, causes chlorotic haloes in infected host leaves (7). *In vitro* the pathogen produces an extracellular toxin which when injected into bean leaves also produces chlorotic haloes (2). Whether the symptoms are caused by infection or by injection of toxin, ornithine accumulates in the chlorotic tissues (9). Here we present evidence which indicates that ornithine accumulation is caused by the inhibition of ornithine carbamyl transferase of the host by the toxin.

Toxin Preparation. The halo blight toxin was partially purified from the culture filtrate of a virulent strain of *P. phaseolicola* grown in the YEP medium (9). Five hundred milliliters of the filtrate were concentrated to 20 ml under reduced pressure at 50 C, and the concentrate was desalted with 10 volumes of methanol. The extract was concentrated and the procedure repeated two more times. The final extract was evaporated to dryness and made up to 10 ml with distilled water. Four milliliters of this sample were applied to a 2- × 45-cm column of Sephadex G-10. The column was eluted with distilled water at a flow rate of 17.6 ml/hr (fraction volume, 2.2 ml). An identical batch of the medium, which was not inoculated, was subjected to the same procedure including incubation under the growth conditions, as a control and is designated nongrown. The culture filtrates are designated grown. Aliquots (25 μl) from every third fraction collected from the Sephadex column were assayed for halo-inducing activity on young trifoliate leaves of 4-week-old Red Kidney bean plants in a growth chamber (2). The same fractions were diluted 10-fold with distilled water and 80 μl of the diluted fraction were used to test its inhibitory activity against OCT[2] of bean leaves. To determine v_e/v_o (retention volume/void volume) of the active material, fractions with halo-inducing activity were concentrated to 2 ml and put on an analytical column (90 × 1.2 cm) of Sephadex G-10. The elution rate was 10.8 ml/hr (fraction volume, 1.8 ml).

Enzyme Preparation. Acetone powder was prepared from trifoliate leaves of 3 to 4 week-old Red Kidney bean plants as described before (4). Five grams of the acetone powder were stirred for 1 hr in 90 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 5×10^{-3} M 2-mercaptoethanol. The slurry was centrifuged at 18,000*g* for 10 min, and the supernatant which contained 10 to 12 mg of protein per ml served as the OCT source. Enzyme assays were made according to the procedure described by Reichard (8). Protein samples were assayed by the biuret method (1).

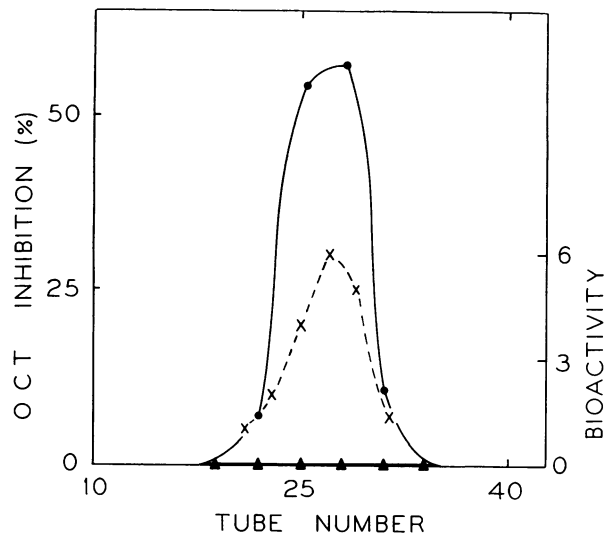

FIG. 1. OCT inhibition and halo-inducing activity of Sephadex G-10 fractions of a methanol-extracted concentrate of culture filtrates of *P. phaseolicola* (grown), and a comparable extract of uninoculated medium (nongrown). Four milliliters of each of grown (●) and nongrown (▲) samples (equivalent to 200 ml of original medium) were applied to a 2- × 45-cm column of Sephadex G-10. Elution was with distilled water at a flow rate of 17.6 ml/hr (fraction volume, 2.2 ml). Twenty-five microliters of each fraction were tested for halo-inducing activity, (×, arbitrary units). Fractions were diluted 10-fold with distilled water and 80 μl samples tested for OCT inhibition.

Aliquots of Sephadex eluant, especially at high concentrations, caused some stimulation of the enzyme activity and this interfered with measurement of the inhibitory activity of the toxin. Since many amino acids are known to enhance the activity of the enzyme (3), the stimulation we observed was thought to be caused by the amino acids or peptides in the medium. Inclusion of 10 μM glutamic acid in all reaction mixtures apparently stimulated the enzyme activity to the maximum and, therefore, the aliquots of

[1] Supported in part by Grant RO1 AI 05638 and Grant RO1 AI 09477 from the National Institute of Allergies and Infectious Diseases. Part of this work was done at the Connecticut Agricultural Experiment Station, New Haven, Connecticut. Hawaii Agricultural Experiment Station Journal Series 1222.

[2] Abbreviation: OCT: ornithine carbamyl transferase.

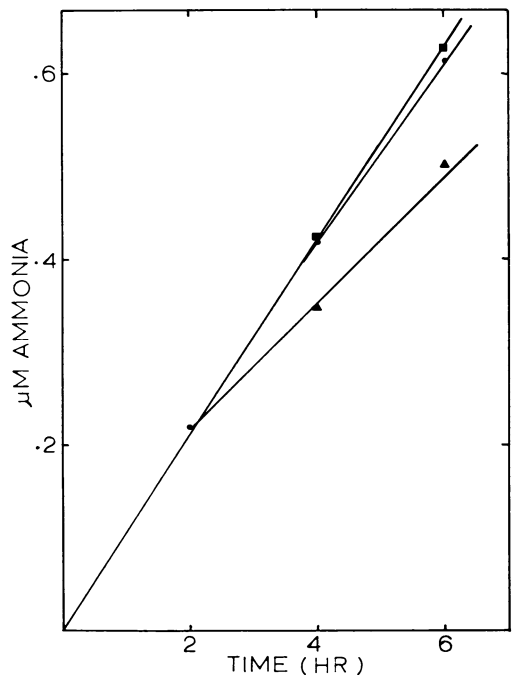

FIG. 2. Toxin used in this experiment was 40 μl of a 10-fold diluted fraction 26 from the experiment shown in Figure 1. The alanine equivalent (after complete hydrolysis) in the diluted fraction 26 was 4 μmoles/ml for grown (▲) and 4.7 μmoles/ml for nongrown (■). Control (●) contained neither fraction. The fractions were added to reaction mixtures 2 hr after enzymatic reaction began.

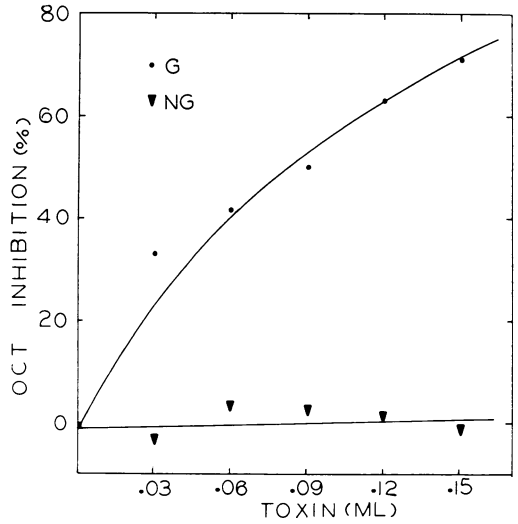

FIG. 3. Effect of toxin concentration on OCT inhibition. ●: G (grown); ▲: NG (nongrown). The fractions tested here were the same as those used in the experiment shown in Figure 2. The duration of assay was 4 hr.

the column fraction did not cause further change in enzyme activity except when the toxin was present. Therefore, 10 μM glutamic acid was routinely included in all enzyme assays.

Results summarized in Figure 1 show that only the halo-inducing fractions showed OCT inhibitory activity. The fractions from nongrown medium neither produced haloes on bean leaves nor inhibited OCT. The v_e/v_o value for the active material as determined on Sephadex G-10 analytical column was 1.95. Time course of OCT (Fig. 2) showed that the reaction rate was rectilinear for at least 6 hr. The rate of reaction was inhibited by about 30% when 40 μl of a 10-fold diluted Sephadex eluant fraction of the grown medium were added to the reaction mixture after 2 hr of incubation without the inhibitor. Corresponding fractions from nongrown medium showed no inhibition of the rate of reaction. Inhibition of OCT was dependent on the concentration of the toxin (Fig. 3). At all concentrations tested the fractions from nongrown medium did not show any OCT inhibitory activity.

Thus, the culture filtrates of *P. phaseolicola* contain an OCT inhibitor which appears to be the same as the halo-inducing agent as indicated by their identical retention volumes on Sephadex gel column. The v_e/v_o ratio shows that the active material was fractionated. It is estimated that 50% OCT inhibition was obtained at a toxin concentration which was 0.5% of that present in the growth medium. Since the toxin appears to be a peptide (S. S. Patil and P. E. Kolattukudy, unpublished), amino acid determination (5) after complete hydrolysis of the toxin fraction (fraction 26 of Fig. 1) used in the experiments shown in Figures 2 and 3 was made. Fifty percent inhibition of the enzyme activity was obtained at about 1.6×10^{-4} M alanine equivalent of the toxin. With 8×10^{-4} M alanine equivalent of the toxin, about 30% inhibition in the rate of reaction was obtained (Fig. 2), in agreement with the results in Figure 3. Since the toxin fractions used in these experiments were still crude, they certainly contained large amounts of peptides from the medium. Therefore, the actual concentration of the toxin in the enzyme assays must have been far less than the concentration indicated above. Thus, the results presented here indicate that the bacteria do produce a potent inhibitor of OCT.

Rudolph and Stahmann (9) showed that injection of the halo blight toxin into leaves produced two effects, a specific effect, accumulation of ornithine, and a nonspecific effect, chlorosis. Ornithine accumulation did not result when chlorosis was induced by other agents. Our results indicate that OCT inhibition is responsible for ornithine accumulation in toxin-treated leaf tissues. How OCT inhibition brings about chlorosis is not clear. Bean plants possess all the enzymes of the ornithine cycle (3), and inhibition of OCT could result in decreased arginine production which in turn may affect the synthesis of enzymes involved in chlorophyll synthesis. Consistent with such a hypothesis, the chlorotic haloes contained almost 50% less arginine as compared to the green tissue (6). Also consistent with the hypothesis is our finding that, when bean leaves were injected with 10 μM citrulline 3 hr prior to or simultaneously with injection with toxin, no chlorotic haloes developed. Obviously, this treatment alleviates the citrulline deficiency which normally results because of OCT inhibition by the toxin. Thus, OCT inhibition appears to be intimately related to development of chlorosis in toxin-treated bean leaves.

LITERATURE CITED

1. GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751–758.
2. HOITINK, H. A. J., R. L. PELLETIER, AND J. G. COULSON. 1966. Toxemia of halo blight of beans. Phytopathology 56: 1062–1065.
3. KLECZKOWSKI, K. AND I. REIFER. 1959. The metabolism of ornithine in green pea, bean and wheat seedlings. Bulletin de L'Academie Polonaise des Sciences. 7: 53–56.
4. KOLATTUKUDY, P. E. 1967. Mechanisms of synthesis of waxy esters in broccoli (*Brassica oleracea*). Biochemistry 6: 2705–2709.
5. MOORE, S. 1968. Amino acid analysis: Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. 243: 6281–6283.
6. PATEL, P. N. AND J. C. WALKER. 1963. Changes in free amino acid and amide content of resistant and susceptible beans after infection with halo blight organism. Phytopathology 53: 522–528.
7. PATEL, P. N. AND J. C. WALKER. 1965. Resistance in *Phaseolus* to halo blight. Phytopathology 55: 889–894.
8. REICHARD, H. 1957. Determination of ornithine carbamyl transferase with microdiffusion technique. Scan. J. Clin. Lab. Invest. 9: 311–312.
9. RUDOLPH, K. AND M. A. STAHMANN. 1966. The accumulation of L-ornithine in halo-blight infected bean plants (*Phaseolus vulgaris* L.), induced by the toxin of the pathogen *Pseudomonas phaseolicola* (Burkh.). Phytopathol. Z. 57: 29–46.