# Inhibition of Ribulose 1,5-Bisphosphate Carboxylase by a Toxin Isolated from *Pseudomonas tabaci*

Leola M. Crosthwaite and S. J. Sheen

Department of Plant Pathology, University of Kentucky, Lexington, KY 40506.
We thank Dr. R. D. Durbin, University of Wisconsin, for the gift of tabtoxin. This research was supported in part by the University of Kentucky Tobacco and Health Research Institute Project 24110 and is published as Journal Series Paper 78-11-99 of the Kentucky Agricultural Experiment Station.
Accepted for Publication 17 October 1978.

## ABSTRACT

CROSTHWAITE, L. M. and S. J. SHEEN. 1979. Inhibition of ribulose 1,5-bisphosphate carboxylase by a toxin isolated from *Pseudomonas tabaci*. Phytopathology 69:376-379.

Toxin purified from culture filtrates of *Pseudomonas tabaci* by column chromatography inhibited ribulose 1,5-bisphosphate carboxylase activity of Fraction I proteins obtained from tobacco cultivars. Inhibition was reduced when toxin was inactivated by heating at 110 C for 20 min. An inverse relationship existed between toxin concentration and inhibition of enzyme activity. When a mixture of toxin and Fraction I protein was fractionated by gel permeation, Fraction I protein showed a reduction of carboxylase activity, suggesting that the protein may be modified by binding with the toxin. On the other hand, the toxin did not inhibit peroxidase and polyphenoloxidase activities from the same tobacco cultivars. A similarly prepared fraction from *P. pisi* did not inhibit the activity of carboxylase and oxidases. The results indicate that *P. tabaci* toxin can inhibit photosynthetic $CO_2$ fixation, and it has a degree of specificity to cellular enzymes; this specificity is not, however, related to resistance or susceptibility of tobacco cultivars to this pathogen.

*Additional key words:* Fraction I protein, tabtoxin.

A number of chlorosis-inducing pathotoxins have been isolated from plant pathogenic pseudomonads. In particular, the agent of tobacco wildfire, *Pseudomonas tabaci*, produces an exotoxin (tabtoxin) that contains a highly reactive $\beta$-lactam ring. Stewart (14) suggested that the mode of action of tabtoxin may involve alkylation of the active site of an enzyme. Glutamine synthetase was implicated as the site of action (11), but recent data with pea glutamine synthetase showed otherwise (9).

Fraction I protein in chloroplasts comprises as much as 50% of the soluble proteins in the leaf and catalyzes both carboxylation and oxygenation of ribulose 1,5-bisphosphate (7). Ribulose 1,5-bisphosphate carboxylase (RuBPCase) is the key enzyme for $CO_2$ fixation in photosynthesis. Any impairment of photosynthetic apparatus at the level of RuBPCase by pathotoxin might result in the degeneration of chloroplast and subsequent development of chlorosis. This article reports the effect of toxin preparations from *P. tabaci* on the RuBPCase activity of tobacco Fraction I protein. Fraction I proteins from tobacco cultivars resistant and susceptible to wildfire pathogen were studied for possible differential responses to tabtoxin. A preliminary report has been published (4).

## MATERIALS AND METHODS

Tobacco, *Nicotiana tabacum* L. 'Burley 21' and 'NC 95,' resistant and susceptible to wildfire, respectively, were grown with hydroponic techniques in a greenhouse. Fraction I protein was isolated from leaves of 2 mo old tobacco plants and recrystallized twice according to the procedure of Chan et al (1). The RuBPCase assay was that of Singh and Wildman (12,13) with slight modifications. Briefly, after heat activation (35 C for 50 min), the solubilized Fraction I protein was preincubated at ambient temperature for 10 min in 10 $\mu$mol of Tris-HCl buffer, pH 7.8, containing 1.75 $\mu$mol of $MgCl_2$, 0.15 $\mu$mol of EDTA, 5 $\mu$mol of $NaHCO_3$, and 1 $\mu$C of $NaH^{14}CO_3$ in a final volume of 0.15 ml. The reaction was started with addition of 14.5 $\mu$mol of ribulose 1,5-bisphosphate (Cal Biochem) in 0.02 ml of the same buffer and stopped after 10 min with 0.15 of 4N HCl. The reaction mixture was dried in a scintillation vial under a stream of air at 60 C, and the residue was resuspended in 1 ml of $H_2O$ and 10 ml of Insta-Gel (New England Nuclear Corp.) before counting with a Packard 3300 liquid scintillation spectrometer.

*P. tabaci* (Kentucky isolate No. 5) was cultured on Woolley's chemically defined medium with aeration at ambient temperature (16). Four days later, the culture solution was centrifuged at 20,000 *g* at 4 C to remove cell debris, and the supernatant fluid was freeze-dried. Crude toxin was prepared by the method of Taylor et al (15) with modifications. The lyophilized culture filtrate was extracted with methanol at 4 C, and the soluble fraction was evaporated in vacuo in the presence of a 1:1 mixture of celite and microcrystallin cellulose (Schwarz/Mann). The powder (1–2 g) was applied to a column (2 × 39 cm) of the same celite and cellulose composition equilibrated with *n*-propanol and water (5:1, v/v). The column was run with 110 ml of *n*-propanol and water (4:1, v/v) followed with *n*-propanol and water (3:1, v/v). An active fraction was eluted off the column with the latter solvent at 560 ml. The collected eluates yielded a fluffy white powder after removal of the solvent in vacuo at 40 C. The toxin preparation was spotted on silica gel thin layer plates and developed with *n*-propanol and water (3:1 v/v). Three ninhydrin positive spots with Rf values of 0.29, 0.26 and 0.20 were detected. A Rf value of 0.24 for tabtoxin analogs in the same solvent system was reported by Taylor et al (15). For comparison, a sample of purified tabtoxin was obtained from Dr. R. D. Durbin of the University of Wisconsin.

Inhibition of RuBPCase activity was determined by mixing the heat-activated Fraction I protein with various amounts of toxin solubilized in the same buffer and maintained at room temperature for 1 hr before the assay. Toxin inactivated at 110 C for 20 min (16) and no toxin in the reaction mixture were the experimental controls. An additional control included a culture of *Pseudomonas pisi* that was fractionated with the same procedures used to prepare *P. tabaci* toxin. *P. pisi* does not produce tabtoxin and is not a pathogen of tobacco.

To test possible binding of toxin with Fraction I protein, each of five vessels containing 2.3 mg of solubilized NC 95 Fraction I protein was added with the following in an equal volume of 25 mM Tris-HCl buffer, pH 7.6: (i) buffer only, (ii) 300 $\mu$g of *P. tabaci* crude toxin preparation, (iii) 300 $\mu$g of heat-inactivated *P. tabaci* crude toxin preparation, (iv) 300$\mu$g of *P. tabaci* crude preparation plus 300 $\mu$g of glutamine, or (v) 300 $\mu$g of glutamine. After 30-min incubation at 25 C, the reaction mixtures were loaded onto separate Sephadex G-25 columns (2 × 13 cm) and eluted with the same

00031-949X/79/000070$03.00/0

TABLE 1. Inhibition of ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity of tobacco Fraction I protein by crude toxin preparations from culture filtrates of *Pseudomonas tabaci* and *P. pisi*[a]

| Cultivar and quantity of Fraction I protein | Treatment and amount of crude toxin | RuBPCase activity (cpm mg of protein) | Inhibition (%) |
|---|---|---|---|
| NC 95 (20 μg) | Control (no toxin added) | 48,132 | 0 |
| | *P. tabaci* toxin (240 μg) | 24,547 | 49 ± 2.1 |
| | *P. tabaci* toxin (24 μg) | 3,369 | 93 ± 9.3 |
| | *P. tabaci* heated toxin (24 μg) | 14,439 | 70 ± 5.6 |
| Burley 21 (20 μg) | Control (no toxin added) | 30,980 | 0 |
| | *P. tabaci* toxin (240 μg) | 10,223 | 67 ± 4.2 |
| | *P. tabaci* toxin (24 μg) | 775 | 98 ± 7.6 |
| | *P. tabaci* heated toxin (24 μg) | 11,995 | 61 ± 4.5 |
| NC 95 (20 μg) | Control (no toxin added) | 71,000 | 0 |
| | *P. pisi* extract (60 μg) | 71,240 | 0 |
| Burley 21 (20 μg) | Control (no toxin added) | 28,844 | 0 |
| | *P. pisi* extract (60 μg) | 28,179 | 2 |

[a]Average of three experiments with four replicates of each treatment. Percent inhibition is expressed as mean ± standard variation, the coefficient of variability in the control experiments was less than 5% in all cases.

TABLE 2. Effect of tabtoxin on ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity of Fraction I protein from tobacco cultivar NC 95

| Toxin dilution[a] | RuBPCase activity (cpm/mg of protein) | Inhibition (%) |
|---|---|---|
| Control (no toxin added) | 43,491 | 0 |
| $1 \times 10^{-2}$ | 45,980 | 0 |
| $2 \times 10^{-3}$ | 33,053 | 24 ± 6.3 |
| $2 \times 10^{-3}$ (heat-inactivated) | 38,272 | 12 ± 2.9 |
| $1 \times 10^{-3}$ | 32,618 | 25 ± 2.8 |
| $5 \times 10^{-4}$ | 28,704 | 34 ± 3.5 |
| $2 \times 10^{-4}$ | 22,180 | 49 ± 2.1 |
| $1 \times 10^{-4}$ | 42,186 | 3 ± 0.1 |

[a]Five microliters of the tabtoxin solution from Dr. R. D. Durbin, University of Wisconsin, was diluted to the given dilution with the assay buffer. Results are average of two experiments with four replications, and the coefficient of variability in the control and $1 \times 10^{-2}$ dilution treatments was less than 5%.

buffer to separate Fraction I protein from the unreacted toxin and glutamine. Fractions immediately following the void volume were collected for assay of RuBPCase activity, which was performed on the basis of 20 μg protein per reaction vessel with four replications for each treatment. Protein quantity was determined by Lowry's method (8) with bovine serum albumin as the standard.

Polyphenoloxidase and peroxidase were prepared from freeze-dried leaves of Burley 21 and NC 95. Leaf powders were ground in cold mortar and pestle in 0.1 M phosphate buffer, pH 7.2, containing 0.1% each of cysteine-HCl and ascorbic acid. After centrifugation at 20,000 *g*, ammonium sulfate was added to the supernatant fluid to a final concentration of 35%. The resultant precipitates contained the polyphenoloxidase fraction. A peroxidase preparation was obtained by adding ammonium sulfate to the 35% supernatant fluid to reach 70% saturation. Both oxidase fractions were dialyzed against 0.05 M phosphate buffer, pH 7.2, for 16 hr at 4 C. The oxidase preparations were lyophilized and their protein content determined by Lowry's method (8). Polyphenoloxidase activity was measured spectrophotometrically as change in absorbance at 475 nm with 3,4-dihydroxyphenylalanine as substrate in 0.1 M phosphate buffer, pH 6.1. Peroxidase was assayed at 485 nm in a mixture of enzyme, $H_2O_2$, and *p*-phenylenediamine in 0.1 M phosphate buffer, pH 6.0. Detailed procedures of oxidase preparation and assay have been reported (10).

## RESULTS AND DISCUSSION

The RuBPCase activity of Fraction I protein treated with the crude toxin preparation of *P. tabaci* was inhibited significantly, compared with that of the control (Table 1). When a high concentration of crude toxin was used, the carboxylase lost nearly one-half of its activity in NC 95 and about two-thirds in Burley 21. However, a tenfold dilution of the crude toxin greatly enhanced the inhibitory effect, and the difference in percent inhibition of two tobacco cultivars became less apparent.

The absence of differential response to wildfire toxin for the Fraction I proteins of resistant and susceptible tobaccos was further substantiated in a separate experiment in which a partially purified toxin (the eluate of ninhydrin positive spots from silica gel thin-layer plates) caused about 50% inhibition of RuBPCase for both cultivars. Yet the amount of partially purified toxin in the reaction mixture containing 20 μg of Fraction I protein was only 50 ng. These results indicate that RuBPCase is not the site for differentiation of resistance and susceptibility to wildfire disease in tobacco. The crude toxin preparation of *P. tabaci* may contain components exhibiting antagonistic effect against the pathotoxin or compounds capable of stimulating RuBPCase activity. Chollet and Anderson (2) reported stimulation of RuBPCase activity by chloroplast metabolites such as NADPH, 6-phosphogluconate, fructose-1,6-bisphosphate, ribulose-5-phosphate and 3-phosphoglycerate when these compounds were preincubated with tobacco Fraction I protein before carboxylase assay. However, a similar preparation from *P. pisi* showed neither stimulation nor inhibition of this photosynthetic enzyme from both tobacco cultivars. Thus, the wildfire toxin may be directly involved in the regulation of photosynthesis or chloroplast function, at least at the level of $CO_2$ fixation.

Inhibition of RuBPCase with purified tabtoxin was consistent with the results of the crude toxin preparation of *P. tabaci* (Table 2). Over the range of $10^{-2}$ to $2 \times 10^{-4}$ dilution, the inhibition showed an inverse relationship. Toxin from the same dilution series produced chlorosis on NC 95 seedling leaves, but the size of chlorotic spots did not clearly correspond to toxin concentrations. Although the $2 \times 10^{-4}$ dilution of the tabtoxin sample inhibited carboxylase about 50%, there was no significant inhibition with a $10^{-4}$ dilution. On a semilogarithmic scale, the inverse relationship with percent inhibition appeared linear (Fig. 1). This pattern of response may stem from a unique property of the enzyme.

It has been proposed that RuBPCase possesses regulatory and catalytic sites for carboxylation; the former requires $Mg^{++}$ and $HCO_3^-$, but the latter involves cofactors and substrates (2,3). In our study, incubation of heat-activated enzyme with tabtoxin may change the conformation of the enzyme that alters the activity of both sites to a different degree. If toxin in high concentration favors

TABLE 3. Effect of crude toxin preparations from *Pseudomonas tabaci* and *P. pisi* on polyphenoloxidase and peroxidase activity

| Cultivar | Treatment[a] | Peroxidase (ΔOD/100 μg of protein/min) | Polyphenoloxidase (ΔOD/mg of protein/min) |
|---|---|---|---|
| | *P. tabaci* toxin only (2 mg/ml) | 0 | 0 |
| | *P. pisi* preparation only (2 mg/ml) | Trace | 0 |
| NC 95 | Oxidase only | 29.51 | 0.12 |
| | Oxidase + *P. tabaci* toxin (2 mg/ml) | 29.80 | 0.12 |
| | Oxidase + *P. tabaci* toxin (0.4 mg/ml) | 29.61 | 0.12 |
| | Oxidase + *P. pisi* extract (2 mg/ml) | 29.80 | 0.12 |
| Burley 21 | Oxidase only | 20.72 | 0.08 |
| | Oxidase + *P. tabaci* toxin (2 mg/ml) | 20.50 | 0.07 |
| | Oxidase + *P. tabaci* toxin (0.4 mg/ml) | 20.50 | 0.08 |
| | Oxidase + *P. pisi* extract (2 mg/ml) | 20.50 | 0.08 |

[a]Oxidases (1 mg/ml) were prepared by dissolving partially purified enzymes in 0.1 M phosphate buffer, pH 6.0.

TABLE 4. Ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity of NC 95 Fraction I protein after treatments with the crude toxin preparation of *Pseudomonas tabaci* and glutamine

| Treatment[a] | RuBPCase activity (cmp/mg of protein) | Inhibition (%) |
|---|---|---|
| Control (no toxin added) | 7,328 | 0 |
| *P. tabaci* toxin | 905 | 88 |
| *P. tabaci* toxin (heated) | 2,351 | 68 |
| *P. tabaci* toxin + glutamine | 6,688 | 9 |
| Glutamine | 7,552 | 0 |

[a]Each reaction mixture contained 2.3 mg of Fraction I protein, 300 μg of crude toxin and/or 300 μg of glutamine.

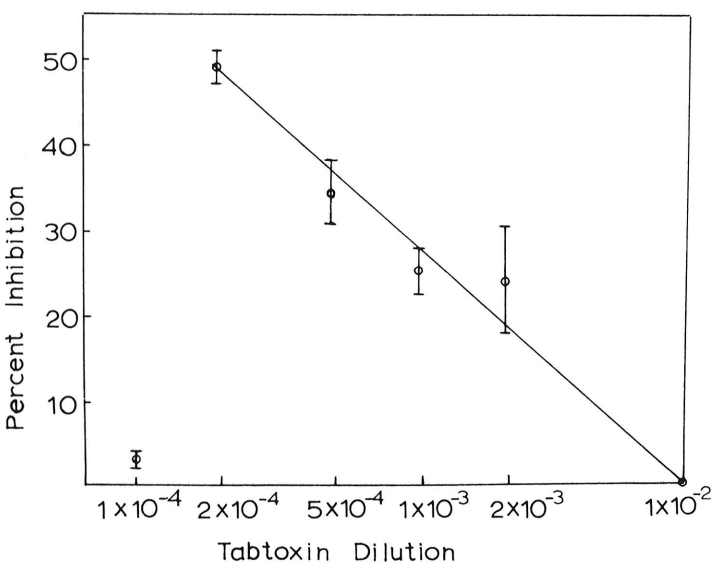


**Fig. 1.** Effect of dilution on percent inhibition of ribulose 1,5-bisphosphate carboxylase by tabtoxin. Each point represents a mean ± standard deviation.

enzyme activation, this might offset its inhibitory effect on carboxylase activity. The pathotoxin of *P. tabaci* may be, therefore, useful as a probe to elucidate the kinetics and molecular mechanism of $CO_2$ fixation by Fraction I protein.

The purified tabtoxin in a $2 \times 10^{-4}$ dilution gave a maximal carboxylase inhibition of about 50% (Table 2). This magnitude of inhibition is comparable to that of the purified *P. tabaci* toxin. On the other hand, the crude toxin preparation exceeded 90% inhibition (Table 1), probably because it consists of a number of active principles including tabtoxin. Durbin et al (6) reported more than one toxin in the culture filtrate of *P. tabaci*. This also may explain why heat treatment caused 20–30% reduction of percent inhibition for the crude toxin but 50% for the purified tabtoxin.

Cytoplasmic oxidases catalyze polyphenol oxidation that leads to tissue necrosis in plants. Inhibition of these oxidases by pathotoxins could result in chlorosis. However, neither *P. tabaci* nor *P. pisi* toxin preparations altered peroxidase or polyphenoloxidase activity (Table 3). Thus, the toxic agents from *P. tabaci* culture filtrate have selective reactions with cellular macromolecules.

The toxin-treated Fraction I protein, which was purified through a Sephadex G-25 column, retained only 12% of the RuBPCase activity of the control (Table 4). Inhibition was reduced by 20% in protein treated with heat-inactivated toxin. The pathotoxic principles from *P. tabaci* likely reacted with RuBPCase rather than with cofactors or substrates since the unreacted toxin was excluded by gel permeation. The inhibitory effect of the toxin was drastically minimized when glutamine was included in the preincubation mixture. These results support the earlier reports that glutamine relieves the toxic effects of tabtoxin in a number of plant and animal species (16). Sinden and Durbin (11) suggested that the mode of action of wildfire toxin was the inhibition of glutamine synthetase. Because tabtoxin failed to inhibit pea glutamine synthetase (9), the mode of action for tabtoxin is uncertain. Tobacco chloroplasts treated with purified tabtoxin showed irreversible structural damage and twofold to fourfold increase in the concentration of ammonia (5). Chloloplast degeneration and release of ammonia indicate the degradation of Fraction I protein. Whether tabtoxin causes the degradation of Fraction I protein in addition to inhibition of carboxylation remains to be elucidated. The possible interactions of other enzymes and macromolecules involved in photosynthesis and photophosphorylation with the toxin also need to be studied.

## LITERATURE CITED


1. CHAN, P. H., K. SAKANO, S. SINGH, and S. G. WILDMAN. 1972. Crystalline Fraction I protein: Preparation in large yield. Science 176:1145-1146.
2. CHOLLET, R., and L. L. ANDERSON. 1976. Regulation of ribulose 1,5-bisphosphate carboxylase-oxygenase activities by temperature pretreatment and chloroplast metabolities. Arch. Biochem. Biophys. 176:344-351.
3. CHU, D. K., and J. A. BASSHAM. 1975. Regulation of ribulose 1,5-diphosphate carboxylase by substrates and other metabolites. Plant Physiol. 55:720-726.
4. CROSTHWAITE, L. M., and S. J. SHEEN. 1977. Inhibition of ribulose 1,5-bisphosphate carboxylase by Pseudomonas tabaci toxin. Proc. Am. Phytopathol. Soc. 4:100.
5. DURBIN, R. D. 1972. Bacterial Phytotoxins: A survey of occurrence, mode of action and composition. In: Phytotoxins in Plant Diseases, ed. R. D. S. Wood, A. Ballio, and A. Graniti. 19-33. N. Y. Academic Press. 530 pp.
6. DURBIN, R. D., T. F. UCHYTIL, J. A. STEELE, and R. De L. D.

RIBEIRO. 1978. Tabtoxinine-β-lactam from Pseudomonas tabaci. Phytochemistry 17:147.
7. KUNG, S. D. 1976. Tobacco Fraction I protein: A unique genetic marker. Science 191:429-434.
8. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193:265-275.
9. PATIL, S. S. 1974. Toxins produced by phytopathogenic bacteria. Annu. Rev. Phytopathology 12:259-279.
10. SHEEN, S. J. 1969. The distribution of polyphenols, chlorogenic acid oxidase and peroxidase in different plant parts of tobacco, Nicotiana tabacum L. Phytochemistry 8:1839-1847.
11. SINDEN, S. L., and R. D. DURBIN. 1968. Glutamine synthetase inhibition: Possible mode of action of wildfire toxin from Pseudomonas tabaci. Nature 219:379-380.
12. SINGH, S., and S. G. WILDMAN. 1973. Chloroplast DNA codes for the ribulose diphosphate carboxylase catalytic site on Fraction I protein of Nicotiana species. Mol. Gen. Genet. 124:187-196.
13. SINGH, S., and S. G. WILDMAN. 1974. Kinetics of cold inactivation and heat reactivation of the ribulose diphosphate carboxylase activity of crystalline Fraction I protein isolated from different species and hybrids of Nicotiana. Plant Cell Physiol. 15:373-379.
14. STEWART, W. W. 1971. Isolation and proof of structure of wildfire toxin. Nature 229:174-178.
15. TAYLOR, P. A., H. K. SCHNOES, and R. D. DURBIN. 1972. Characterization of chlorosis-inducing toxin from a plant pathogenic Pseudomonas sp. Biochem. Biophys. Acta 286:107-117.
16. WOOLLEY, D. W., R. B. PRINGLE, and A. C. BRAUN. 1952. Isolation of the phytopathogenic toxin of Pseudomonas tabaci, an antagonist of methionine. J. Biol. Chem. 197:409-417.