PHASEOTOXIN A: AN ANTIMETABOLITE FROM PSEUDOMONAS PHASEOLICOLA

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SUMMARY

Phaseotoxin, the exotoxin of the bean pathogen Pseudomonas phaseolicola, has been resolved into four biologically active fractions by DEAE cellulose chromatography. Phaseotoxin A, the toxin in the first fraction decomposed to give glutamic acid and inorganic orthophosphate only, and was chromatographically identical with synthetic N-phosphoglutamic acid. Like phaseotoxin A, the synthetic compound inhibits ornithine carbamoyltransferase and induces chlorosis in bean leaves. To our knowledge this is the first report of occurrence of an N-phosphorylated primary amine in nature.

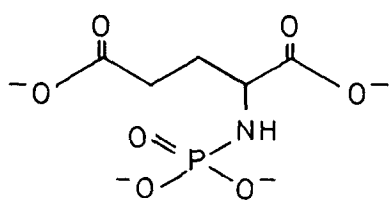
INTRODUCTION

For over 25 years plant pathologists have known that the culture filtrate of Pseudomonas phaseolicola Burk (Dowson), an incitant of the halo blight disease of beans (Phaseolus vulgaris L.), mimicks the pathogen by producing symptoms of chlorosis when injected into bean tissues (1). Whether chlorosis is caused by inoculation of host leaves by the pathogen (2) or by injection of toxic filtrate, large quantities of ornithine accumulate in the affected bean tissues (3). Tam and Patil (5) and Patil et al (6) reported that the active principle of P. phaseolicola, phaseotoxin (1), is a potent and specific inhibitor or ornithine carbamoyltransferase E.C.2.1.3.3, affecting the carbamoylphosphate site rather than the ornithine site of the enzyme. Furthermore, the phaseotoxin-induced chlorosis in bean leaves can be reversed by the administration of citrulline and arginine, but not other naturally-occurring amino acids, indicating that OCT inhibition may

be the cause of chlorosis in affected bean tissues (6).

Phaseotoxin also appears to be involved in the virulence of the pathogen for its host. In inoculated resistant cultivars of bean the pathogen grows substantially at first but soon the growth is impeded as the host responds by producing the hyper-sensitive reaction (HR) (7). However, in spite of this substantial bacterial multiplication no toxin is detected in such tissues (8). In inoculated susceptible cultivars no HR occurs and sustained growth of the pathogen results. Unlike infected resistant tissues, however, large quantities of phaseotoxin are detected in infected susceptible tissues (8). Patil and Gnanamanickam (9) recently reported that when resistant cultivars are supplied with phaseotoxin before inoculation with the pathogen, the HR is suppressed and bacterial multiplication comparable to or greater than that in susceptible tissue occurs. Moreover, the accumulation of antibacterial isoflavonoids, which normally accompanies the HR in inoculated resistant tissues, is severely curtailed when resistant tissues are treated with phaseotoxin. Thus, phaseotoxin seems to be involved in the establishment of the pathogen in its host.

Here we report the separation of phaseotoxin into four biologically active fractions (A, B, C, D) by DEAE cellulose chromatography (4) and the identification of the toxin in fraction A as N-phosphoglutamic acid. This is the first report of isolation of this novel phosphoroamide from a biological source.



MATERIALS AND METHODS

The culture filtrates were concentrated and the salts were precipitated with methanol as previously described (4). The active fractions obtained after gel filtration on a column of Sephadex G-10 were combined and subjected to ultrafiltration at 4° on a Diaflo UM-2 membrane (Amicon Corp.) to separate the toxin from impurities of high molecular weight (>1000). The filtrate, which contained the phaseotoxin, was desalted and freed from the inactive material of low molecular weight (>500) by ultrafiltration on a Diaflo UM-05 membrane.

The retentate from the latter ultrafiltration in 2 ml of water, containing approximately 150,000 units* of toxin, was applied to a column of alkali washed DEAE cellulose (1.2 cm x 21 cm) which had been previously equilibrated with deionized water. After washing the column with 65 ml of deionized water, the toxin was eluted with a linear gradient (0.0 to 0.03 M) of aqueous NaCl. Fractions were monitored for ornithine carbamoyltransferase inhibitory activity as previously described (5) and phosphate concentration in fractions was determined by the method of Lazarus and Chou (10).

N-phosphoglutamic acid was synthesized as follows. The p-toluenesufonate salt of dibenzyl glutamate, prepared from glutamic acid, benzyl alcohol, and p-toluenesulfonic acid using a procedure similar to that described for the preparation of the benzene-sufonate salt of benzyl glycinate (11), was reacted with dibenzyl chlorophosphonate, prepared by oxidizing dibenzyl hydrogen phosphate (12) with chlorine in chloroform, to form the tetrabenzylester of N-phosphoglutamic acid. This latter material was dissolved in 50% methanol-water, excess $NaHCO_3$ and 10% Pd/C catalyst were added, and the mixture was hydrogenated. Hydrogenolysis was allowed to proceed for 3 hr and the water-soluble portion of the product was assayed for inhibitory activity against ornithine carbamoyltransferase.

RESULTS AND DISCUSSION

The elution profile of the biological activity, shown in Figure 1, indicated that ion exchange chromatography on DEAE cellulose separated the phaseotoxin into two well resolved (peaks A and B) and two poorly resolved (peaks C and D) toxins. Fractions which comprised peak A were combined, exhaustively desalted by ultrafiltration on UM-05, and freeze-dried. Analysis of the residue from peak A by pmr and cmr spectroscopy revealed the presence of only glutamic acid. When the sample was tested for bioactivity after completion of the nmr work, no

*one unit of toxin is that amount of toxin which inhibits the activity of ornithine carbamoyltransferase by 50% under standard assay conditions (5).

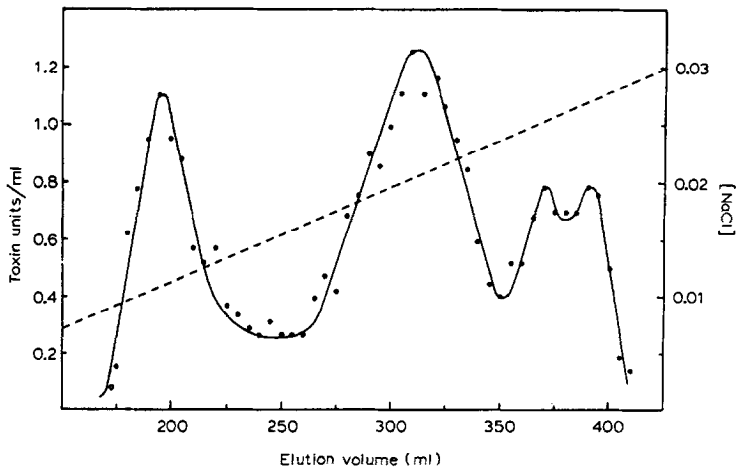


Fig. 1. DEAE cellulose gradient chromatography of phaseotoxin. Two ml of a partially purified preparation of phaseotoxin containing 150,000 units of toxin were applied to a column of DEAE cellulose (1.2 x 21 cm) equilibrated with deionized water. After washing (15 ml/hr, 2.5 ml/fraction) the column with 65 ml of deionized water, it was connected to a linear gradient of NaCl (0.0 - 0.03 M) and eluted at the same rate. Fractions were assayed for ornithine carbamoyltransferase (OCT) inhibitory activity as described (5).

enzyme inhibitory activity could be detected.

Combustion analysis revealed the presence of an appreciable residue in the sample which was subsequently shown to be inorganic phosphate. Before we could determine the pmr and cmr spectra of the phaseotoxin in peak A, it had decomposed. We therefore concluded that L-glutamic acid and phosphate were products of decomposition and that phaseotoxin A might be N-phosphoglutamic acid especially since N-phosphoaminoacids are reported to be very labile (13) and have never been properly characterized.

The concentration of phosphate was much higher than the amino acid content in the decomposed sample indicating that the original toxin was contaminated with extraneous phosphate. In order to separate the extraneous phosphate from the active

compound an undegraded sample of phaseotoxin A, which had been exhaustively desalted by ultrafiltration on UM-05, was applied in 2 ml of deionized water to a 1.2 x 95 cm column of Biogel P-2 (- 400 mesh) equilibrated in deionized water. The column was eluted with water at 7.6 ml/hr and fractions were assayed for enzyme inhibitory activity and phosphate.

The phosphate elution profile showed three poorly resolved peaks; only the substances in the first two peaks, however, had ornithine carbamoyltransferase inhibitory activity. The third peak was inorganic phosphate. The fractions from the first two peaks were combined, concentrated, and rechromatographed on Biogel P-2 as described above. The elution profile of the enzyme inhibitory activity for the recycled material is shown in Figure 2a. Since only a very small amount of material was recovered after recycling the active fractions, no attempt was made to assay for glutamate or phosphate. (Because of extremely low yields and because of the instability of Phaseotoxin A we have not been able to accumulate adequate quantities of the compound). To examine the behaviour of synthetic N-phosphoglutamic acid on the Biogel P-2 column a solution of this material in 2 ml of water (containing approximately 1000 units of activity) was chromatographed on a Biogel column as described above. Analysis of fractions for phosphate showed four peaks, the elution volumes of the first two peaks being identical to those obtained from Biogel chromatography of natural phaseotoxin A. Analysis of acid hydrolyzates of peaks 1 and 2 for glutamate showed that only peak 2 contained glutamate. The fractions for the first two peaks were combined and recycled on the same Biogel column. The elution profile of the OCT activity is shown in Fig. 2b and there was a complete coincidence between biologi-

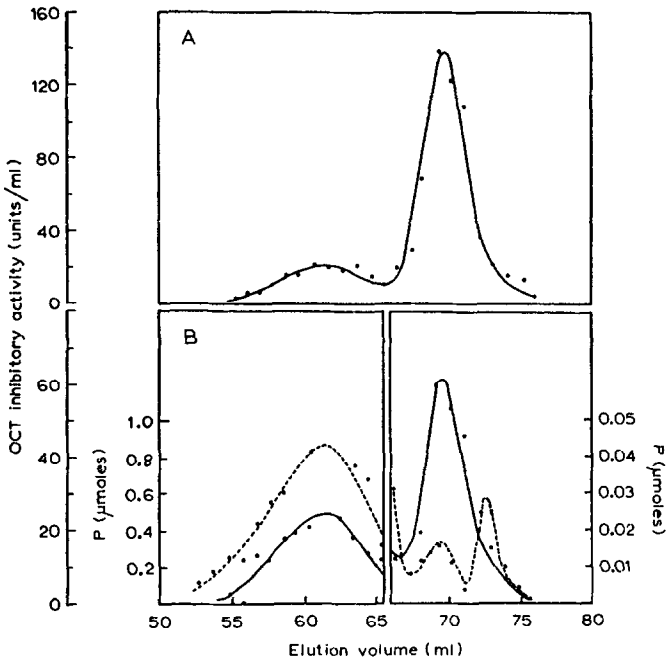


Fig. 2. Fractionation of phaseotoxin A (2a) and of the synthetic N-phosphoglutamic acid (2b) on Biogel P-2 (1.2 x 95 cm) at 6°C. Two ml of either phaseotoxin A containing 1500 units or N-phosphoglutamic acid containing 1000 units were applied to the column equilibrated in deionized water. Elution was at 7.6 ml/hr and 1.0 ml fractions were collected. Fractions were examined for ornithine carbamoyltransferase inhibition (5) and for phosphate (10).

o—o, enzyme inhibitory activity;

o-----o, Phosphorus.

cal activity and phosphate content. The specific activities for the substances in peak 1 and peak 2 were 27 units/μmole phosphate and 4725 units/μmole phosphate, respectively. Since the material in peak 1 contained phosphate, but no glutamate, and was found to have the same elution volume as pyrophosphate, we concluded that this fraction was either pyrophosphate or a polyphosphate. Analysis showed that peak 2 contained phosphate and glutamate in roughly a 1:1 ratio.

Comparison of the synthetic material with the natural product showed that it had the same elution volume when subjected to DEAE cellulose gradient chromatography as the natural product (phaseotoxin A). Also, both phaseotoxin A and N-phosphoglutamic acid* induced chlorosis in treated bean leaves.

Several amino acids were found in the acid hydrolyzates of fractions B, C and D (Fig. 1), among them glycine (15). To determine if the phosphoroamide of glycine is also biologically active we synthesized N-phosphoglycine by catalytic hydrogenolysis of tribenzyl N-phosphoglycinate in aqueous sodium bicarbonate. The pmr spectrum of tribenzyl N-phosphoglycinate in CCl_4 exhibits a doublet of doublets at δ 3.52 for the glycinate methylene protons (J = 12 Hz for coupling to phosphorus and J = 7 Hz for coupling to the adjacent NH proton). The smaller coupling was removed by deuterium exchange. After desalting the reaction mixture by ultrafiltration on UM-05, the retentate was freeze-dried to give N-phosphoglycine. The proton magnetic resonance (pmr) spectrum of the N-phosphoglycine in D_2O exhibited a doublet at δ 3.50 (J = 10 Hz) for the methylene protons. Chemical shifts are reported in δ units using p-dioxane (δ 3.80 relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate, δ 0) as an internal reference in D_2O. As the nmr sample stood over-night in the presence of acid, hydrolysis of the compound occurred as shown by disappearance of the doublet and appearance of singlet for glycine. N-Phosphoglycine inhibits ornithine carbamoyltransferase, however, it

*Unpublished work by H.B.F. Dixon (Department of Biochemistry, University of Cambridge) and S.S. Patil showed that a more direct synthesis of N-phosphoglutamic acid, (from glutamic acid and $POCl_3$) made by a modification of the method of Winnick and Scott (14) likewise gave a biologically active compound.

has only 1/3 the specific activity (activity per μmole phosphate) of that of N-phosphoglutamic acid.

Several N-phosphorylated primary amines including N-phosphoglutamic acid are known to chemistry (13, 14). However, as far as we could determine this is the first report of occurrence of an N-phosphorylated primary amine in nature. Its structure and its ability to act as a specific inhibitor of the carbamoylphosphate site of ornithine carbamoyltransferase (5) indicate that N-phosphoglutamic acid acts as an analog of carbamoylphosphate. Only one other compound of this type, (not found in nature) N-phosphonomethylglycine (15) is biologically active. Its mode of action is still unknown but it has been commercially used as a potent herbicide. The presence of glycine in semipurified phaseotoxin (16) and the ability of synthetic N-phosphoglycine to inhibit ornithine carbamoyltransferase indicate that compounds in Peaks B, C and D may also be N-phosphoroamides.

There are five known toxigenic Pseudomonad pathogens which attack five different species of plants (17). P. tabaci, the pathogen of tobacco, produces two novel β-lactams, tabtoxins (18, 19), as does P. coronafaciens, the pathogen of oats (17). P. phaseolicola apparently produces completely different toxins, a mixture of N-phosphoroamino acids which we have called phaseotoxins. Interestingly, the toxins of P. glycinea and P. tomato, the pathogens of soybean and tomato, are reported to be chromatographically and biologically similar to the phaseotoxins (20).

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