Hydrolysis of tabtoxins by plant and bacterial enzymes

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Summary. Tabtoxins, dipeptides that induce chlorosis in plants, are produced by several closely related phytopathogenic *Pseudomonas* spp. Culture filtrates from these bacteria and extracts prepared from leaves of their hosts contain multiple enzymes that hydrolyze the peptide bond of the toxins.

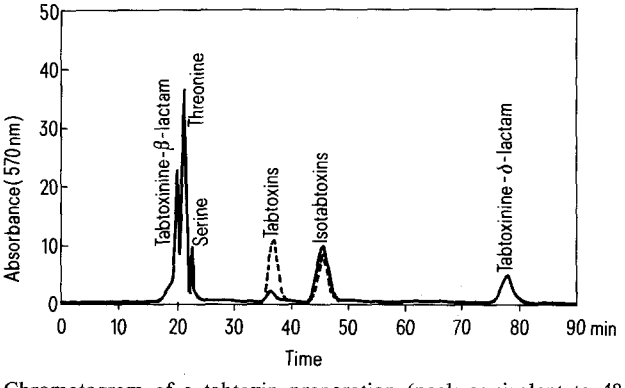
The phytopathogenic bacterium *Pseudomonas tabaci* (Wolf and Foster) Stevens and several closely related species produce toxins that cause chlorosis in higher plants[1] and inhibit the growth of *Escherichia coli* (Migula) Castellani and Chalmers (Durbin, unpublished data). Called tabtoxins, they are dipeptides containing tabtoxinine-β-lactam [2-amino-4-(3-hydroxy-2-oxo-azocyclobutan-3-yl)-butanoic acid] linked to either threonine or serine[2,3]. Originally it was postulated that these toxins acted as antimetabolites of methionine, partially because of the similarity in activity of the toxins to methionine sulfoximine (MSO), which at that time was also thought to affect methionine metabolism[4]. Later investigations showed that although methionine would compete with MSO for passage across the plasmalemma[5], its actual site of action is glutamine synthetase (GS)[6]. Initial experiments with crude preparations of the tabtoxins and GS supported the idea that the tabtoxins also inhibited GS[7].

However, in further studies using purified components and a sensitive assay[8], no inhibition of GS could be demonstrated (Durbin and Uchytil, unpublished data). More recently, free tabtoxinine-β-lactam has been found in culture filtrates of *P. tabaci*[9]. Most interestingly, as with the tabtoxins, this compound causes chlorosis, and, as with the toxins, the reaction is light dependent and there is an accumulation of ammonia in chlorotic tissue[10]. Furthermore, chlorosis is always evident earlier than with the tabtoxins. These findings support the hypothesis that tabtoxinine-β-lactam could play a key role in vivo in chlorosis production. If so, the peptide bond of the tabtoxins would have to be cleaved. The present studies provide evidence that both plants and *Pseudomonas* spp. have enzymes with peptidase activity capable of hydrolyzing the tabtoxins, and discusses the possible significance of these findings to the mechanism of action.

Materials and methods. Peptidase activity of bacterial culture filtrates was tested by growing 5 isolates of *P. tabaci* and 3 isolates of *P. coronafaciens* (Elliott) Stevens for 5 days with agitation in Woolley's medium[11] at 26 °C. Cells were removed by centrifugation (12,000 × g for 15 min), sodium azide added to give a final concentration of 0.05%, and the filtrate sterilized (Millipore, 0.45 µm). A portion of the filtrate was maintained at 4 °C and the remainder incubated at 37 °C for 12 h. Additional controls consisted of adding 0.01% diethylpyrocarbonate (v/v), a histidine-modifying reagent[12], to the filtrate incubated at 37 °C, or adjustment of filtrate from pH 7.3 to 2 or 10. After incubation, aliquots from each treatment with and without the addition of the tabtoxins and their hydrolytic products[2] were chromatographed on an amino acid analyzer. Leaves from species that are hosts of tabtoxin-producing pseudomonads (tobacco, bean, pea, oat, corn and timothy) were also examined for peptidase activity. The tissue (17 g) was ground for 1 min in a Waring blendor in 150–200 ml. 0.1 M Tris, pH 7, containing 0.01% 2-mercaptoethanol (v/v) and 6 g Poly Clar AT. The suspension was filtered, centrifuged and a 40–70% ammonium sulfate fraction prepared in 0.01 M Tris, pH 7, containing 0.01% dithiothreitol (v/v). After washing with 3 vol. of buffer (24 ml) in the Amicon chamber, the tabtoxins were introduced and the extent of hydrolysis was assayed as described above. The same procedure was used with diseased tobacco, *Nicotiana tabacum* L. 'Havana 38' 5 days after inoculation with *P. tabaci* (ATCC 11528). A 70% ammonium sulfate precipitate prepared from a culture filtrate of *P. tabaci* (ATCC 11528) was suspended in 0.01 M Tris buffer, pH 7, and electrophoresed on a 7% acrylamide gel slab. The slab was sliced transversely into 5 sections, each section ground in a Duall homogenizer to a slurry and extracted 3 times with 0.1 M Tris buffer, pH 7. The combined extracts were then washed and concentrated to 1 ml in a 10 ml Amicon chamber using a UM-10 membrane (10,000 mol. wt exclusion limit). Tabtoxins were introduced into the chamber and incubated with stirring for 1 h at room temperature. The effluent was collected by N_2 pressure and an aliquot chromatographed on the amino acid analyzer. A similar analysis for peptidase activity was done using a 40–70% ammonium sulfate fraction from tobacco leaf tissue.

Intercellular fluids[13] were obtained from healthy and diseased leaves of tobacco that had been inoculated 5 days previously with either of 2 isolates of *P. tabaci,* of which one did not hydrolyze the tabtoxins. The fluids were concentrated 20-fold, washed and incubated with the tabtoxins at room temperature for 12 h in the presence of 0.05% sodium azide. Aliquots were removed and analyzed for hydrolysis of the tabtoxins.

Direct evidence for the in vivo conversion of the tabtoxins was sought by treating tobacco leaves with purified, ^{14}C-labelled tabtoxins. They were introduced into the leaves by lightly pricking a leaf through a series of 10 µl droplets containing the toxins. After letting the droplets dry, additional 10 µl droplets of water were placed on the same spots and allowed to dry. The leaf surface was then thoroughly washed. In this way about 50% of the original material was taken up by the plant. After 36 h incubation at 28 °C in the dark, 12 mm diameter leaf discs were cut out, immediately frozen in dry ice, ground in a Duall homogenizer, then extracted twice using cold 70% ethanol and 1% TCA. The



Chromatogram of a tabtoxin preparation (peak equivalent to 48 nmoles glycine) before (dotted line) and after (solid line) incubation for 12 h at 37 °C with a culture filtrate of *P. tabaci* (ATCC 11528).

extract was concentrated 20-fold and an aliquot chromatographed on the amino acid analyzer using a stream splitter attachment. Fractions constituting a single, radioactive peak were pooled and individually cochromatographed with authentic samples of the tabtoxins and their hydrolytic products.

Results. Culture filtrates from isolates of *P. tabaci* and *P. coronafaciens,* with the exception of 1 isolate of each species, were capable of hydrolyzing tabtoxins when incubated at 37 °C but not 4 °C (figure). Addition of diethylpyrocarbonate or adjustment of the pH to 2 or 10 abolished peptidase activity. The 40–70% ammonium sulfate fraction from all the plant species and diseased tobacco also hydrolyzed the tabtoxins. However, preparations of the intercellular fluids from both healthy and diseased tobacco leaf tissues had no detectable activity.

Analysis of gel electrophoretograms of the bacterial and plant preparations showed that hydrolysis was due to multiple enzymes; every section of the gels exhibited activity. When duplicate gel sections were stained with Coomassie brilliant blue, discrete protein bands became visible, indicating that the protein separations were satisfactory. All bacterial and plant preparations having peptidase activity on the tabtoxins were inactive on the isotabtoxins. The same result was obtained with leucine aminopeptidase. Most ($\geq 85\%$) of the radioactivity after incubation of the ^{14}C-tabtoxins in tobacco leaves was still associated with the tabtoxins or their products. It was distributed among these compounds as follows: tabtoxinine-β-lactam, threonine and serine, 8.5%; tabtoxins 75%; isotabtoxins 6%; and an unknown compound or compounds, 11%, eluting 11 min after tabtoxinine-δ-lactam. A sterile solution of ^{14}C-tabtoxins maintained in buffer, pH 7, at 28 °C for 36 h did not have any hydrolysis products when similarly analyzed. The same result was also obtained when ^{14}C-tabtoxins and a tobacco leaf preparation were processed immediately after mixing.

Discussion. Collectively, these results strongly suggest that the tabtoxins are hydrolyzed during disease development. Most likely this occurs in the plant cells, possibly within the vacuole[14], by the action of multiple enzymes having peptidase activity. Hydrolysis of the tabtoxins also could occur in the intercellular spaces by bacterial and/or plant action but no evidence to support this notion was obtained.

Hydrolysis of tabtoxins appears to be of biological significance because our initial experiments have shown that tabtoxinine-β-lactam inhibits GS, whereas the tabtoxins do not. If further detailed work substantiates GS as the primary site of action, it would mean that hydrolysis is a prerequisite for activity. A chlorosis-inducing toxin produced by *P. phaseolicola* (Burkh.) Dowson, phaseolotoxin, has already been reported to be hydrolyzed by plant enzymes; however, phaseolotoxin itself is as active an inhibitor of ornithine carbamyl transferase (OCT) as one of the hydrolytic products, Nδ-(phosphosulphamyl) ornithine[15]. Thus, it does not appear that in this case hydrolysis is mandatory for biological activity, assuming that OCT is the primary site of action and that the OCT preparation did not hydrolyze phaseolotoxin.

How widespread the occurrence might be of metabolic activation of phytotoxins is currently unknown. However, many are potential candidates having structures containing hydrolizable bonds or groups that are readily modified[16,17]. We suggest that the metabolic products of toxins could, if they are also toxic, have different affinities or even different sites of action from the parent compound(s). More emphasis should be given to examining the metabolism of phytotoxins as part of studies on their mechanism of action.

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Simple, geometrical model for a typical echinocyte III

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Summary. The purpose of the present work is to obtain information relative to the volume and surface area of echinocyte, type III, on the basis of simple, micrographical evidence. A concrete case has been examined as direct demonstration of theory and techniques of calculus.

It is obvious that it is impossible to know certain geometrical data, as the surface area, relative to any spatial structure (cells, organs or other bodies) without some theoretical information and functional hypotheses, concerning the shape and dimensional ratios of the body in question.

In other words the evaluation of a particular kind of geometrical data implies the adoption of an appropriate model, on the basis of known linear parameters (lengths, diameters, axial ratios, etc.) strictly speaking the only geometrical data, which one may measure, i.e. obtained by means of a direct, empirical determination.

Having stated this, we present a typical situation, for which