Plant Physiol. (1968) 43, 683–686

Studies on the Mode of Action of Tomatine as a Fungitoxic Agent[1]

P. A. Arneson[2] and R. D. Durbin

U.S.D.A. Pioneering Research Laboratory, Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706

Received November 20, 1967.

Abstract. **Removal of 1 or more sugar residues from the α-tomatine molecule markedly decreased its fungitoxicity. While partial hydrolysis of α-tomatine did not greatly affect its surfactant properties, it did destroy the ability of this alkaloid to form a complex with cholesterol. Only unprotonated α-tomatine was capable of binding cholesterol; the protonated form did not. Since α-tomatine was far more toxic at a high pH than at a low pH, this suggests that the unprotonated alkaloid is the active form and that it acts by complexing with fungal sterols.**

Tomatine (fig 1) is a steroidal alkaloid found in a number of *Solanum* and *Lycopersicon* species. The natural product, α-tomatine, is a glycoside containing D-xylose, D-galactose, and 2 molecules of D-glucose. It can be partially hydrolyzed in dilute acid to yield β_1-tomatine (minus xylose), β_2-tomatine (minus 1 glucose), γ-tomatine (minus xylose and 1 glucose) and tomatidine (the aglycone) (6).

Alpha-tomatine is toxic to a broad range of fungi (4, 12). However, *Septoria lycopersici,* incitant of tomato leaf spot, can detoxify α-tomatine by enzymically removing 1 glucose unit, leaving β_2-tomatine (2). This raises an interesting question concerning α-tomatine's action as a fungitoxic agent: what property or properties, lost upon hydrolysis, are responsible for its toxicity?

At present, the mode of action of tomatine and related glyco-alkaloids is not well established.

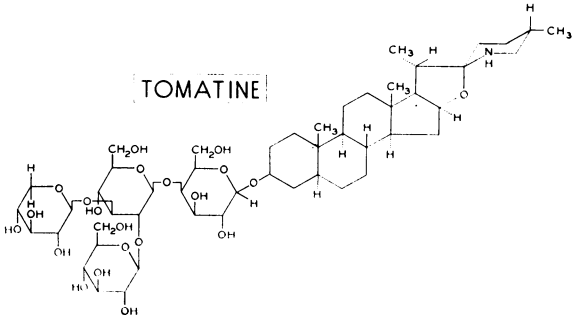

FIG. 1. α-Tomatine: O-β-D-xylopyranosyl-(1→3)-[O-β-D-glucopyranosyl-(1→2)] O-β-D-glucopyranosyl-(1→4)-O-β-D-galactopyranosyl-tomatidine.

McKee (7), observing that zoospores of *Phytophthora infestans* disintegrated in solanine solutions, suggested that this was due to the surfactant properties of the glyco-alkaloids. Owing to the hydrophobic steroid moiety at 1 end of the molecule and the hydrophilic carbohydrate moiety at the other, α-tomatine does possess surfactant properties, but whether they can account for the toxicity of the molecule has not been demonstrated. Alternatively, Schreiber (8) suggested that the toxicity of several steroidal glyco-alkaloids, including α-tomatine, toward insects was due to the ability of these compounds to bind sterols. Steroids having a free 3β-hydroxyl group do form a 1:1 molecular complex with tomatine which is quite stable and can be dissociated only in concentrated acid solutions (10).

This study of α-tomatine and its hydrolysis products was undertaken to determine whether their fungitoxicity could be related to their surfactant properties or to their ability to form complexes with cholesterol.

Materials and Methods

The Toxicity of α-Tomatine and its Hydrolysis Products. The minimum concentration of α-, β_1- and β_2-tomatine and tomatidine[3] required to completely inhibit mycelial growth of *Colletotrichum orbiculare, Septoria linicola,* and *Helminthosporium turcicum* was determined using the channel test of Wolters (11). The alkaloids were dissolved in 1 equivalent of 10 mM HCl, and serial dilutions containing 1×10^{-2}, 2×10^{-3}, 4×10^{-4}, 8×10^{-5},

[1] Work supported by National Science Foundation Grant No. IN-35F.

[2] Present address: Division of Tropical Research, Tela Railroad Company, La Lima, Honduras.

[3] α-tomatine was obtained from Sigma Chemical Company, St. Louis, Missouri. β_1- and β_2-tomatine and tomatidine were obtained by refluxing the α-tomatine in 0.1 M HCl for 2 hours and chromatographically separating the resulting alkaloids on an alumina column eluted with ethanol:water (9:1, v/v) (1).

and 16 × 10^{-6} M alkaloid were prepared. The solutions were autoclaved (hydrolysis was negligible under these conditions) and 1 ml aliquots were aseptically pipetted into each channel. After 5 days at 24° the mycelial growth at the edge of the channel was measured to the nearest 0.1 mm. Four replicate measurements for each fungus were made for each dilution of each alkaloid. The mean growth was then plotted versus the negative logarithm of the molar concentration of the alkaloid. The resulting straight line was extrapolated to zero growth or in other words, 100% inhibition, and that concentration defined as the MC_{100}.

The Effect of pH on the Toxicity of α-Tomatine. Portions of potato broth (filtrate from 250 g peeled, diced potatoes/liter tap water steamed for 30 min) were adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 with 1 M HCl or NaOH. Serial dilutions of α-tomatine in potato broth at each pH were prepared to give molar concentrations of 2.5 × 10^{-3}, 5 × 10^{-4}, 1 × 10^{-4}, 2 × 10^{-5}, and 4 × 10^{-6}. The solutions were then autoclaved, and to 2 ml of each dilution at each pH was added 0.5 ml of a spore suspension (about 10^6 spores/ml) of *H. turcicum*. The percent germination (germ tubes at least as long as the spore) was determined after 20 hours incubation at 24° on a rotary shaker.

The Surfactant Properties of α-Tomatine and its Hydrolysis Products. Solutions of hydrochlorides of α-, β_1-, and β_2-tomatine and tomatidine were prepared by dissolving the alkaloids in 1 equivalent of 20 mM HCl and diluting with distilled water to the desired concentrations (at the higher concentrations β_1- and β_2-tomatine were in a colloidal suspension rather than a solution because of their low water solubility). Solutions of the unprotonated alkaloids were prepared by grinding the powdered alkaloids to a paste in a small amount of water and then diluting them to the desired concentration (in all cases these solutions contained undissolved solids in colloidal suspension). The surface tensions of aqueous solutions of a known fungitoxic surfactant, dodecylguanidine acetate (dodine), were also determined. The surface tension of each solution at 22° was measured by the height to which it would rise in a capillary tube. The surface tension, γ, was calculated from the following equation:

$$\gamma = \text{one-half } hdgr$$

where h is the height to which the liquid rises, d is the density of the liquid, g is the acceleration due to gravity and r is the radius of the capillary tube.

Complexing of Cholesterol by α-Tomatine. Ethanol solutions of α-, β_1-, and β_2-tomatine and tomatidine free bases were prepared, each containing 2 mM alkaloid. A similar solution was prepared of α-tomatine hydrochloride. A 1 mM solution of cholesterol in acetone:diethylether (1:1, v/v) was prepared containing sufficient ^{14}C-labeled cholesterol to bring the radioactivity of the solution to about 10^5 dpm/ml.

At time zero, 10 ml of the radioactive cholesterol solution was added to 10 ml of each of the alkaloid solutions with rapid mixing. At 10 minute intervals, 3 ml of the mixture was withdrawn with a syringe and quickly pressed through a paper filter in a Swinney adapter into a test tube. Aliquots of the filtrate were then pipetted into vials of scintillation fluid (3.0 g PPO, 0.18 g dimethyl POPOP, 560 ml toluene and 38 ml absolute ethanol) and the radioactivity determined with a liquid-scintillation spectrometer.

To confirm the presence or absence of a complex, which might have escaped detection by failing to precipitate, the reaction mixtures remaining after 1 hour incubation were analyzed by thin-layer chromatography. About 50 μl of each mixture was spotted on plates of Silica Gel G. The chromatograms were developed with 95% ethanol:ethyl acetate:diethylamine (15:5:1, v/v/v). Blue-gray spots appeared after the chromatograms were sprayed with a saturated solution of ceric sulfate in 65% sulfuric acid and heated to 110° for 10 minutes.

Results

The Toxicity of α-Tomatine and its Hydrolysis Products. The minimum concentrations of α-, β_1-, β_2-tomatine and tomatidine required to completely inhibit mycelial growth of *C. orbiculare*, *S. linicola*, and *H. turcicum* are given in table I. Although the hydrolysis products were all less toxic than α-tomatine, their relative toxicity varied for each fungus. For example, with *C. orbiculare* about 5 times as much β_1-tomatine, 4 times as much β_2-tomatine and 3 times as much tomatidine as α-tomatine were required to completely inhibit mycelial growth, whereas in the case of *S. linicola* 25 times as much β_1-tomatine, 32 times as much β_2-tomatine and

Table I. *Fungitoxicity of α-Tomatine and Its Hydrolysis Products*

	Colletotrichum orbiculare	*Septoria linicola*	*Helminthosporium turcicum*
		MC_{100} (mM)[1]	
α-Tomatine	2.0	0.4	0.13
β_1-Tomatine	10	10	5.0
β_2-Tomatine	8.0	13	4.0
Tomatidine	6.3	790	50

[1] The MC_{100} is the minimum concentration of alkaloid required to completely inhibit mycelial growth.

nearly 2000 times as much tomatidine as α-tomatine were required for complete inhibition of growth.

The Effect of pH on the Toxicity of α-Tomatine. LD_{50} values at each pH were determined by probit analysis. These values were then plotted versus pH (fig 2). About 300 times more α-tomatine was required at pH 3.0 to give the same inhibition as at pH 8.0.

Assuming that the only effect of pH was on the protonation of the alkaloid, that the unprotonated alkaloid was the only toxic form of α-tomatine and that at pH 8.0 α-tomatine was completely dissociated into the unprotonated form, theoretical LD_{50} values were calculated for each pH. The LD_{50} at

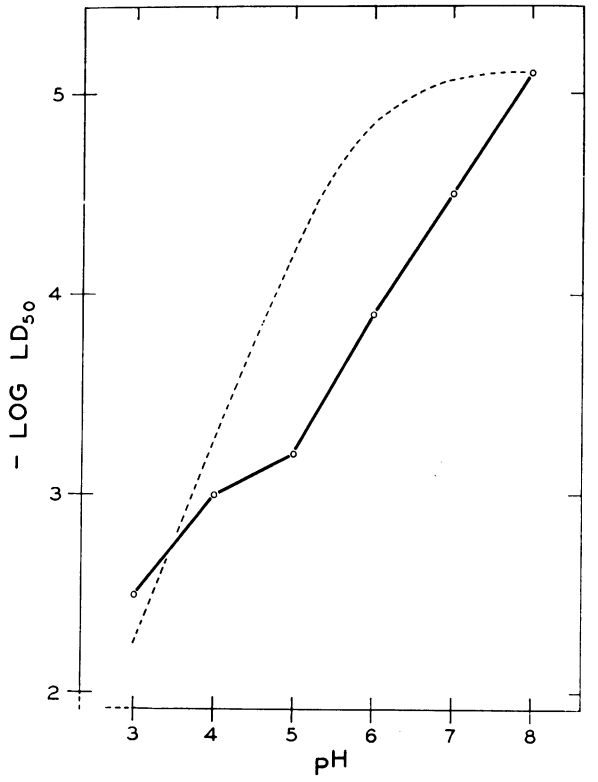

FIG. 2. Effect of pH on the inhibition of spore germination by α-tomatine. The —log LD_{50} values were plotted versus pH. The dotted line shows the theoretical curve which would be obtained if the pH effect were due only to the protonation of the alkaloid and if the free base were the only toxic form of α-tomatine.

pH 8.0 was taken as the true value for unprotonated α-tomatine, and the theoretical values then calculated from the amount of unprotonated alkaloid present, as determined from the titration curve of α-tomatine (1). These values are represented by the dotted curve in figure 2.

The Surfactant Properties of α-Tomatine and its Hydrolysis Products. The surface tensions of colloidal suspensions of the unprotonated alkaloids in water are plotted as a function of concentration

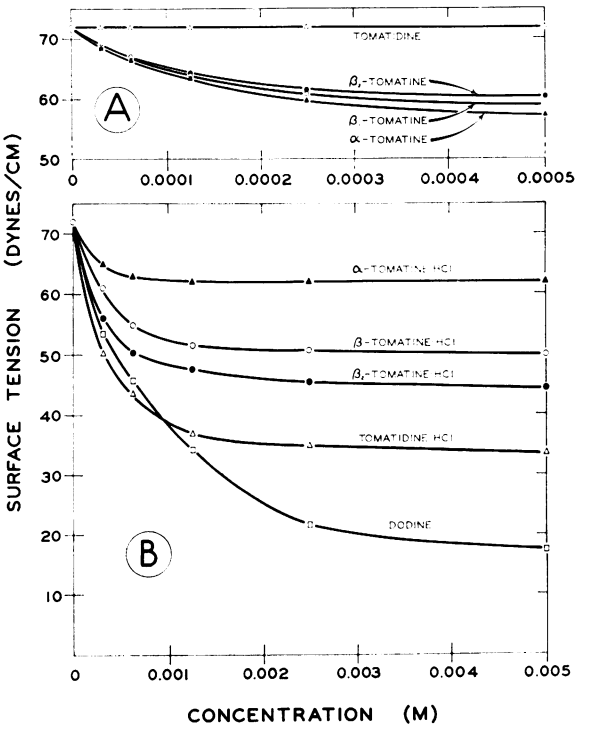

FIG. 3. Surface tensions of aqueous solutions of α-tomatine and its hydrolysis products. A) The unprotonated alkaloids. B) The alkaloid hydrochlorides.

in figure 3A. Minimum values were approached at 0.5 mM which for α-tomatine was 58.6 dynes/cm, β_1-tomatine, 59.8 dynes/cm, β_2-tomatine, 60.4 dynes/cm, and tomatidine, 71.5 dynes/cm. The surface tension of pure water was 72.0 dynes/cm.

The surface tensions of aqueous hydrochloride solutions are plotted in figure 3B. The surface

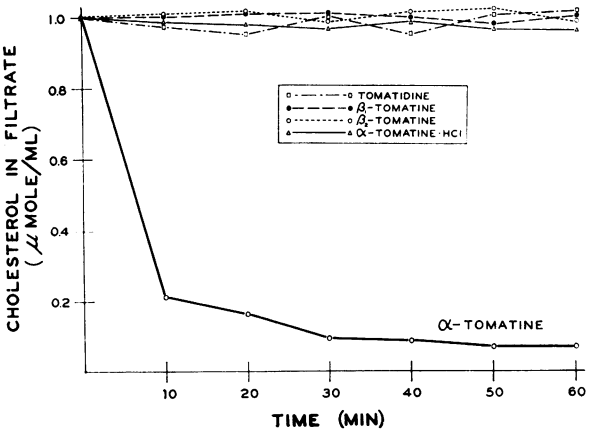

FIG. 4. Precipitation of cholesterol by α-tomatine and its hydrolysis products. A solution of cholesterol-^{14}C in acetone:diethyl ether was mixed with an ethanol solution of the alkaloid. At 10 minute intervals, aliquots were withdrawn and filtered. The radioactivity of the filtrate was then measured.

tensions approached a minimum value at 5 mM for all these compounds. At this concentration, the surface tensions were: α-tomatine•HCl, 62.3 dynes/cm; β_1-tomatine•HCl, 50.0 dynes/cm; β_2-tomatine•HCl, 44.4 dynes/cm; tomatidine, 33.6 dynes/cm; and dodine, 17.8 dynes/cm.

Complexing of Cholesterol by α-Tomatine. The concentration of cholesterol remaining in solution after reaction with α-tomatine and its hydrolysis products is plotted as a function of time in figure 4. Only unprotonated α-tomatine was capable of precipitating cholesterol; α-tomatine hydrochloride, β_1-tomatine, β_2-tomatine, and tomatidine were not. Analysis by thin-layer chromatography confirmed the presence of a cholesterol-α-tomatine complex, but showed no evidence of complexes with the other compounds.

Discussion

The toxicity of α-tomatine is greater than can be accounted for by its surfactant properties alone. Both the protonated and unprotonated forms cause only relatively small depressions of the surface tension of water. Furthermore, dodine, although a better surfactant, is less toxic than α-tomatine; the MC_{100} of dodine for *H. turcicum* is 2 mM compared to 0.1 mM for α-tomatine.

Removing sugars from the molecule does affect its surfactant properties. In the unprotonated form, the hydrolysis products of α-tomatine are slightly less polar than α-tomatine itself and thus are poorer surfactants. However, this hardly seems sufficient to account for the difference in toxicity between α-tomatine and β_2-tomatine. In acid solutions, removing sugars from α-tomatine actually makes it a better surfactant since the protonated amino group is far more hydrophilic than the tetrasaccharide, and when both are present at opposite ends of the molecule they tend to oppose each other in their effect on the molecule's surfactant properties.

The fungitoxicity of tomatine is more closely correlated with its ability to complex with sterols. Hydrolysis of any of the sugars from α-tomatine eliminates its ability to form a complex with cholesterol and at the same time markedly reduces its fungitoxicity. Furthermore, only unprotonated α-tomatine will complex with cholesterol; the protonated form will not. This is consistent with the observation that α-tomatine is far more toxic at a high pH than at a low pH, although the effects of pH on toxicity appear to be more complex than can be explained entirely by protonation of the amino group (fig 2).

Further evidence for a sterol-complexing mode of action of α-tomatine is the observation that although α-tomatine is toxic to many fungi, species of *Pythium* and *Phytophthora* are relatively insensitive to it (1). These fungi are also insensitive to other known sterol complexing agents, such as the polyene antibiotics (3, 5), presumably because their membranes do not contain sterols. Compounds which bind sterols do disrupt cell membranes as shown by their hemolytic activity (9). The effect of α-tomatine may be to react with the membrane sterols and thus alter membrane permeability.

Despite the apparent correlation between fungitoxicity and the ability to complex with sterols, the mode of action of α-tomatine as a fungitoxic agent will remain open to question until certain anomalies can be explained. No evidence was found of complexing between cholesterol and the hydrolysis products of α-tomatine. Nevertheless, these compounds were still slightly toxic. Indeed, Wolters (12) reported that tomatidine was even more toxic to some fungi than α-tomatine itself. Perhaps there is more than 1 mode of action responsible for the fungitoxicity of these alkaloids.

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