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SELECTIVE TOXINS AND ANALOGS PRODUCED BY HELMINTHOSPORIUM SACCHARI: PRODUCTION, ISOLATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY

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SELECTIVE TOXINS AND ANALOGS PRODUCED BY HELMINTHOSPORIUM SACCHARI: PRODUCTION, ISOLATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY

By

Robert Stanley Livingston

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ABSTRACT

SELECTIVE TOXINS AND ANALOGS PRODUCED BY
HELMINTHOSPORIUM SACCHARI:
PRODUCTION, ISOLATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY

By

Robert Stanley Livingston

A method was developed to isolate the host-selective toxins and toxin analogs from cultures of Helminthosporium sacchari. The procedure include the use of activated charcoal, ion exchange, gel, and flash chromatography, plus reverse phase HPLC. HS toxin was characterized in part by NMR, MS, derivatization and degradative chemical techniques. A structure proposed by other workers was shown to be wrong. Toxins contain two chains of β-1,5 linked galactofuranose units attached to an unsymmetrical sesquiterpene. The three forms of toxin (A, B, and C) differed in relative abilities to induce electrolyte loss from susceptible sugarcane tissues.

In cultures of the fungus, toxin concentration peaked at three weeks, followed by a rapid decline. H. sacchari was found to produce a β-galactofuranosidase which removes galactose units from toxin, thus producing lower molecular weight analogs (toxoids). Twenty one different toxoids were produced by sequential removal of galactose from the three forms of toxin. Each of the three toxins and six of the toxoids with three galactose units were partially digested with enzyme; the resulting toxoids were separated by HPLC and the arrangement of galactose units was determined.

Finally the biological activities of the toxoids were determined. One of the toxoids with three galactose units proved to be toxic to some

but not all H. sacchari-susceptible clones of sugarcane. This toxoid was as toxic to certain sensitive sugarcane clones as was the most active form of the toxin (four galactose units). The other toxoids were non-toxic on all other tested clones of sugarcane. All isomers of the toxoids with three galactose units gave protection against action of the toxin; the three galactose toxoids were more effective than were the toxoids with two galactose units. Toxoids with only one unit of galactose provided very little protection. Other experiments showed that the sesquiterpene isomer, the number of galactose units, and the arrangement of galactose units in the molecule all play a significant role in determining toxicity (as determined by induction of electrolyte loss) and relative ability to counteract the effects of toxin on sugarcane tissue.

ACKNOWLEDGEMENTS

I would like to thank Robert Scheffer, Keisuke Kohmoto, Mark Lesney and Steven Briggs for their support, guidance and friendship.

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GENERAL INTRODUCTION

The disease of sugarcane known as "eyespot", caused by Helminthosporium sacchari, became severe in Hawaii, Puerto Rico, Java, and elsewhere when certain new cultivars were planted on a large scale (5). The major symptoms of the disease include "eyespot" lesions on leaves, with subsequent development of a necrotic lesion which can advance to the leaf tip. The fungus is easily isolated from the eyespot lesions, but usually is absent from the "runner" lesion above the eyespot. This situation was interpreted by Lee and other early workers to mean that a toxin, produced by the fungus in the eyespot, is transported up the leaf. Lee made some detailed early studies but came to no conclusions regarding the toxin (6). Lee's preparations appeared to be slightly more toxic to the susceptible than to the resistant clone of sugarcane, but his data were inconclusive. He suggested that the toxic component in culture filtrates of H. sacchari was a nitrite.

Conclusive evidence for the presence of a toxin was presented in 1971 (8). The toxin was partially purified by a procedure involving solvent extraction and gel permeation chromatography. Many clones of sugarcane were tested for their reaction to the pathogen and to the toxin; those clones resistant to the pathogen were insensitive to the toxin and clones susceptible to the pathogen were sensitive to the toxin. Toxin was shown to be a small molecule which, when partially purified, could reproduce some of the symptoms of natural infection.

Many papers on HS toxin have been published since it was first shown that filtrates of H. sacchari are selectively toxic to susceptible sugarcane. In fact, the disease and the toxin involved have become an important model case for studies on the molecular basis of plant disease development. In 1971 Steiner and Strobel published a paper containing a proposed structure for the toxin which was given the name helminthosporoside (9). The proposed structure was 2-hydroxycyclopropyl-α-D-galactopyranoside. A series of papers followed, from which was developed a theory which explained the high degree of specificity and the mode of toxicity of the toxin (11). In 1975 a summary of this work was presented in "Scientific American" (12). The theory involved a receptor or toxin-binding protein in sensitive tissue; resistant tissue was said to contain a similar protein which did not bind toxin. Toxin binding caused a change in the conformation of the receptor and the surrounding lipids of the membrane. These changes induced the activation of a K^{+}-Mg^{++}ATPase. The result was an unbalanced flow of ions in the cell, a breakdown in normal cell functions, and eventual death of the cell. Several subsequent papers have been published which support and yet always change the details of the theory (2,3). However, a critical analysis of the work of Strobel et al. was published (1), and attempts in our laboratory to repeat the critical experiments were unsuccessful (7).

My initial work was directed toward obtaining a purified preparation of the toxin produced by H. sacchari (HS toxin). The purified toxin was then characterized (in part) by spectral and chemical techniques; the original characterization by Steiner and Strobel (9) was shown to be wrong. These aspects of my work are presented as dissertation section I, which was published with the title "Isolation and characterization of

host-selective toxin from Helminthosporium sacchari". During the course of this work, an enzyme was discovered which hydrolyzes the toxin molecule, freeing galactose units. This work is described in section II of the dissertation, which was published with the title "Conversion of Helminthosporium sacchari toxin to toxoids by β-galactofuranosidase from Helminthosporium". Next, analogs of the toxic compound were discovered, these were characterized and shown to be inhibitors of the toxic effect of HS toxin. This part of the work was prepared as a manuscript for publication, and is given as section III of the dissertation; it was accepted for publication with the title "Toxic and protective effects of analogs of Helminthosporium sacchari toxin on sugarcane tissues". Finally, twenty-one non-toxic analogs of HS toxin were isolated and their protective effects against toxin were determined. One of the analogs proved to be as toxic as is HS toxin, but only to certain clones of sugarcane. This part of the research was described in a manuscript that was prepared for publication; it is presented as section IV of the dissertation.

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EXPERIMENTAL I

ISOLATION AND CHARACTERIZATION OF HOST-SELECTIVE TOXIN FROM *HELMINTHOSPORIUM SACCHARI*:

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Isolation and Characterization of Host-selective Toxin from *Helminthosporium sacchari**

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Helminthosporium sacchari **infects certain clones of sugar cane and produces a toxin with the same plant selectivity as the fungus itself. The toxin was purified by use of activated charcoal plus thin layer, gel, and ion exchange chromatography. Gas chromatography (GC) of a trimethylsilyl derivative of toxin gave a single peak. Toxin was characterized by GC, mass spectroscopy (MS), and NMR spectroscopy. The spectra of hydrolytic products showed that toxin contains galactose plus a $C_{15}H_{21}$ moiety which appears to be a sesquiterpene. Spectral data and methylation procedures showed that toxin contains an oligosaccharide composed of β, 1 → 5 galactofuranose units (probably 5 units). Several interconvertible forms of the $C_{15}H_{21}$ moiety were evident after acid hydrolysis. Toxin was separated from 3 closely related, nontoxic compounds ("noxins"), which contained galactose plus the $C_{15}H_{21}$ moiety. Comparative data show that the toxin examined in this study is the same as the toxin described by Steiner and Strobel (Steiner, G. W., and Strobel, G. A. (1971) *J. Biol. Chem.* 246, 4350–4357). The data also show that the previously proposed structure is incorrect.**

At least 15 plant-infecting fungi are now known to produce substances with selective toxicity against susceptible hosts. Such toxins are not active against non-host species, and against host genotypes that are resistant to the fungus. Several of these "host-selective toxins" have been isolated and partially characterized (6). However, only the toxin from *Alternaria mali* affecting certain apple cultivars has been characterized completely (4), the structure confirmed (8), and the molecule synthesized (2). *A. mali* toxin is a cyclic depsipeptide with a M_r 445.

Helminthosporium sacchari (Van Breda de Haan) Butler selectively parasitizes some cultivars (clones) of sugar cane, causing a disease known as "eyespot." Several years ago, the fungus was shown to produce a toxin with selective effects which matched those of the fungus. Steiner and Strobel (7) isolated the toxic compound and characterized it as 2-hydroxycyclopropyl-α-D-galactopyranoside (trivial name, helminthosporoside). The proposed structure has not been confirmed. Nevertheless, this and other work on *H. sacchari* toxin is often cited in discussions of the molecular basis of disease development and disease resistance in plants (1).

We have re-examined the toxin from *H. sacchari*. Characterization is not yet complete, but we feel that the data should be published because of the importance of the work (7) and the controversies involved (10). An abstract describing some of our work was published (3).

MATERIALS AND METHODS[1]

RESULTS

Water-soluble Hydrolytic Products of Toxin—The aqueous phase of acid-hydrolyzed toxin was chromatographed on thin layer plates, using several different solvent systems, with diphenylamine:aniline:phosphoric acid as the indicator reagent. The R_F values and color reactions of the resulting spots matched those of galactose standards. The water-soluble fraction was then derivatized with Tri-Sil-Z and subjected to GC-MS[2], using columns containing several different liquid phases. Retention times for peaks from gas chromatography matched those of derivatized galactose (α, β, and γ forms); mass spectra confirmed the presence of galactose but there was no indication of other sugars.

Chloroform-soluble Hydrolytic Products of Toxin—The chloroform phase of hydrolyzed toxin was subjected to gas chromatography, using a column (1.8 m) packed with OV-1 (3%) and a temperature of 170°C. Four major peaks and several minor peaks were observed (Fig. 1). Each major peak was later characterized by MS as a 15-carbon compound; for convenience, they are identified as C-15a, C-15b, C-15c, and C-15d (Fig. 1). Enriched preparations of the four major C-15 products were made by TLC followed by chromatography with an LH-20 column (see "Materials and Methods").

Possible interconversion of the C-15 products was considered. Aliquots of each of the 4 major C-15 products in aqueous trifluoroacetic acid (0.1, 0.05, and 0.01 M) were held at 95°C for 2.5 h. The solutions were then extracted with 3 equal volumes of chloroform and the combined extracts were subjected to GC. Results showed that each of the major C-15 products gave at least trace quantities of the others. For example, when C-15c was exposed to acid at 0.05 M, GC

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[1] Portions of this paper (including "Materials and Methods," some of the "Results," Fig. 5, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-1305, cite author(s), and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: GC, gas chromatography; MS, mass spectroscopy; Me_2SO, dimethyl sulfoxide; Me_3Si, trimethylsilyl; TLC, thin-layer chromatography; EI/D, electron ionization subsequent to thermal desorption from field emitters.

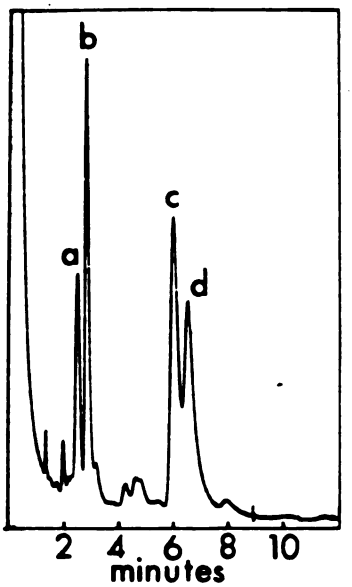

FIG. 1. **Gas-liquid chromatogram of the chloroform-soluble hydrolytic products of toxin from *Helminthosporium sacchari*.** The four major products had retention times of 2.53, 2.85, 5.95, and 6.47 min. Several other products were present in smaller amounts. All products had an apparent molecular ion in the mass spectrum at *m/e* 218; high resolution peak matching indicated an empirical formula of $C_{15}H_{21}OH$.

showed the presence of C-15d, plus two products with retention times very similar to those of C-15a and -b, plus two other products (retention times, 4.22 and 4.58 min). These data indicate that the C-15 products are unstable and are interconvertible. Available data do not establish which form of the C-15 moiety is in the toxin molecule; indeed, toxin might exist as isomers based on different forms of the C-15 moiety.

Mass Spectroscopy—The following conditions were used for intact toxin, using the Hewlett-Packard instrument with the direct probe electron impact method: source temperature was 200°C, probe was heated from ambient to 280°C, ionization voltage was 35 eV, and ions were monitored from *m/e* 35 to 500. The spectrum (Fig. 2) showed the highest visible mass ion at 380. The Varian CH-5 mass spectrometer was used for high resolution peak matching; ionization was by electron impact with accelerating voltage at 70 eV. Peak matching with *m/e* 380 indicated that the most probable empirical formula was $C_{21}H_{32}O_6$. The low resolution spectrum had a peak at *m/e* 201, ($C_{15}H_{21}$) which appears to be $C_{21}H_{32}O_6$ minus galactose. High resolution peak matching on the *m/e* 201 confirmed this empirical formula. There was a third peak at *m/e* 217; peak matching indicated that this was $C_{15}H_{21}O$, a carbon-hydrogen compound plus oxygen from the galactoside linkage. A peak at *m/e* 259 was predicted; this should be $C_{17}H_{23}O_2$ ($C_{15}H_{21} + C_2H_2O_2 \rightarrow C_{17}H_{23}O_3$, or a carbon-hydrogen unit plus a portion of galactose, a known break for the galactoside linkage). The low resolution spectrum had the expected peak at *m/e* 259, and peak matching confirmed the predicted formula.

Intact toxin was dissolved in D_2O (99.7%) to exchange deuterium for the hydroxyl protons. The mass spectrum of deuterium-labeled toxin should show an increase of one atomic mass unit/exchangeable proton. Results showed that *m/e* 380 was shifted to *m/e* 384, indicating four exchangeable protons. Related *m/e* values were shifted comparably. Again,

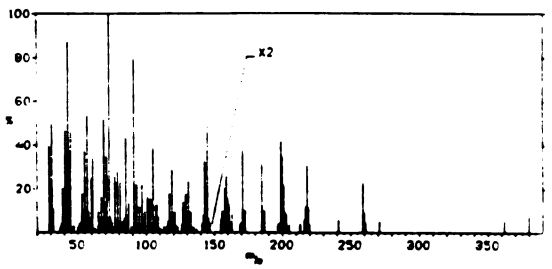

FIG. 2. **Mass spectrum of toxin from *Helminthosporium sacchari*.** Host-selective toxin (5 μg) was inserted into the source (temperature 200°C) by direct probe and ionized by electron ionization (35 *ev*). The probe was heated from 25 to 280°C (30°C/min); toxin was emitted at 280°C. Ions were monitored from *m/e* 29 to 500 on a Hewlett-Packard 5985 *A* mass spectrometer.

these data are consistent for a structure containing galactose, plus a unit containing 15 carbons with hydrogen ($C_6H_{11}O_6 + C_{15}H_{21} \rightarrow C_{21}H_{32}O_6$), with the 15-carbon unit attached to the galactose at a single position. This would leave four free hydroxyl groups which would exchange protons for deuterium. The mild conditions required for hydrolysis (with release of galactose and a 15-carbon unit from intact toxin), plus the MS data, suggest a galactosidic linkage.

Mass spectra for methylated toxin were obtained with the Varian CH-5 spectrometer, using the EI/D method. Acceleration voltage was 1.0 kV, which gives increased sensitivity and lower accuracy (±1.0 *m/e*) with masses >1000. Filament current was 18 mA, and ions were monitored up to *m/e* 1150, which is maximum for the spectrometer. The spectrum showed ions at *m/e* 1060 and 1093, indicating that toxin contains at least 5 galactose units plus a $C_{15}H_{21}$ moiety.

The four chloroform-soluble, 15-carbon hydrolytic products of toxin (C-15, a–d) were characterized by GC-low resolution MS and by high resolution peak matching. Peak matching of the *m/e* 201 fragment indicated that the empirical formula was the same as that determined for the ion at *m/e* 201 ($C_{15}H_{21}$) in the spectrum of intact toxin. Spectra were collected in both electron impact and chemical ionization (methane) modes; these data indicated that 218 was the probable molecular mass for all 4 products. Peak matching of the *m/e* 218 produced the empirical formula $C_{15}H_{21}OH$. The C-15 products may be sesquiterpene-type compounds, with a single hydroxyl formed during hydrolysis, and with 5 points of unsaturation (double bonds or ring structures). All four C-15 hydrolytic products produced a similar fragmentation pattern (see Fig. 5 in miniprint), with variation in the relative abundance of the several fragments. This indicates that the C-15 compounds are very similar in structure, possibly differing only in the position of double bonds. The conclusion is supported by proton NMR data on the C-15 breakdown products (given below).

Most ions in the mass spectrum of toxin were also present in the C-15 moiety. Only a few ions were from galactose, which is not surprising because sugar moieties are known to give weak mass spectra. Peaks for intact toxin at *m/e* 43, 73, and 91 can be attributed to both the galactose and the C-15 fragment (overlapping ions were confirmed by high resolution mass spectroscopy). The ions at *m/e* 60 and 61 are typical of galactose and were not found in the spectra of the C-15 moiety. These sets of ions were emitted from the probe at the same high temperature, indicating that the toxin preparation is a single, large, relatively nonvolatile compound.

Proton NMR Studies—The NMR spectrum of toxin in D_2O contains 14 major peaks or distinct regions (Fig. 3, Table I).

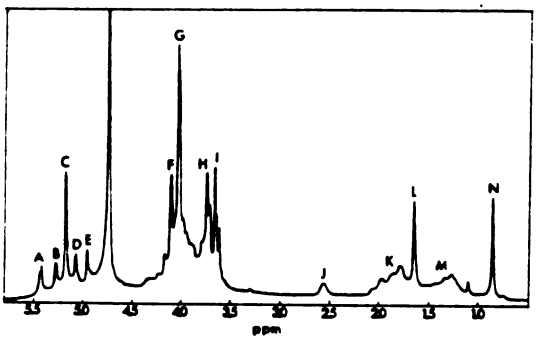

FIG. 3. **Proton NMR spectrum of toxin at 180 mHz.** Host-selective toxin (10 mg) from *Helminthosporium sacchari* was dried under reduced pressure and rinsed several times with D_2O to remove H_2O. The sample was dissolved in 0.5 ml of D_2O (99.7% deuterium) in a 5-mm NMR tube. The Fourier transformed spectrum was obtained from 25 transients on a Bruker WH-180 instrument. The chemical shifts in ppm for the major *peaks* are given in Table I.

TABLE I

Proton NMR spectrum of toxin from Helminthosporium sacchari: shift position and number of protons for each peak

Peak[a]	Shift position[b]	Number of protons[c]	Peak[a]	Shift position[b]	Number of protons[c]
	ppm			*ppm*	
A	5.41	1	H	3.74	—[d]
B	5.77	1	I	3.66	—[d]
C	5.17	3	J	2.57	1
D	5.06	1	K	2.0–1.7	8
E	4.96	1	L	1.64	3
F	4.10	—[d]	M	1.5–1.2	7
G	4.03	—[d]	N	0.85	3

[a] See Fig. 5.
[b] Calculated from the HDO peak at 4.74.
[c] Determined from the area under each peak.
[d] Peaks F, G, H, and I represent a total of 38 protons.

The spread of the seven peaks in the 4.91 to 5.41 ppm region is too great to result from splitting of a single group of protons. Therefore, these peaks represent five groups of protons. One group at 5.17 ppm contains three identical protons; the other peaks in this region represent one proton each. These peaks probably are from olefinic protons. Strong absorbance at 3.6 to 4.2 ppm represents approximately 35 to 40 protons on carbons with a hydroxyl group; most of this absorbance is from protons in galactose. This number of protons suggests as many as 5 or 6 galactose units/molecule of toxin. β anomeric protons also absorb in the low field end of the 3.6 to 4.2 ppm region. The peaks at 4.03 ppm may be from galactose protons, but protons on the C-15 moiety that are involved in the galactoside linkage could also absorb in this region. A single C-15 unit/molecule of toxin probably has no more than two protons absorbing in the 4.03 region; this is not enough to account for the large peak at 4.03 ppm. Another possibility could be more than one C-15 moiety/toxin molecule.

Proton NMR studies of the isolated C-15 moieties showed that they varied in the number of olefinic protons (2 to 4). There was a sharp singlet upfield, which represents an isolated methyl group. The sharp singlet at 4 ppm was assigned to the two protons on the carbon with the hydroxyl, which probably is adjacent to a carbon with a double bond.

The NMR spectrum of the Me_3Si derivative of toxin, in deuterated chloroform, was dominated by absorption at 0.25 to 0.05 ppm. The area of this absorption was proportional to the number of protons on those Me_3Si groups that had replaced each hydroxyl group on the original toxin. The two peaks at 1.64 and 0.85 ppm, each representing 3 protons, were used to determine the units of area/proton. To determine the number of Me_3Si groups/toxin molecule, the number of protons in the 0.25 to 0.05 region (125 to 150 protons) was divided by the number of protons/Me_3Si group (9 protons). There were 14 to 17 Me_3Si groups/molecule of toxin, indicating 5 galactose units.

^{13}C NMR Studies—The dominant characteristic of the ^{13}C NMR spectrum of intact toxin was the strong intensity of peaks from 63.9 to 85.5 ppm (Fig. 4). These peaks are from carbons in galactose; they are much more intense than the peaks given by the C-15 moiety. The difference in intensity of the galactose and the C-15 peaks is great enough to suggest that there are several galactose units per C-15 moiety in the toxin molecule. The six to eight peaks from 116.7 to 151.2 ppm indicate six to eight olefinic carbons. These data indicate the presence of three double bonds and two ring structures per C-15 fragment, suggesting a sesquiterpene. This is consistent with the empirical formula predicted by mass spectral peak matching data. The region of the spectrum in which aliphatic carbons absorb (18 to 48.6 ppm) contains 9 to 13 peaks. The carbon atom of the C-15 moiety which shares an oxygen with the oligosaccharide will absorb in the same region as does galactose (63.9 to 85.5 ppm).

The peak at 63.9 ppm is very close to the assigned shift position for the C-6 of a galactofuranoside (63.6 ppm). The shift position of a C-1 is characteristic for α and β anomeric forms of pyranosides and furanosides. The lack of peaks at 101–104 ppm rules out α- and β-galactopyranosides and α-galactofuranoside (9). The peak at 109.8 ppm indicates a β-linked galactofuranoside (5).

Mass Spectroscopy of Helminthosporoside—MS data on helminthosporoside from G. Strobel (Montana State University) were taken for comparison with our preparation of toxin

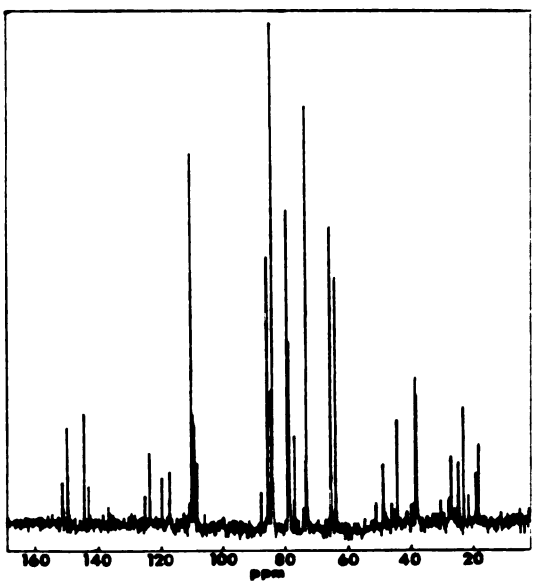

FIG. 4. **^{13}C NMR spectrum of toxin at 45.2 mHz.** Host-selective toxin (approximately 90 mg) from *Helminthosporium sacchari* was dissolved in D_2O in a 10 mm NMR tube. The Fourier-transformed spectrum was obtained from 17,034 transients. The sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid was used as an external reference.

from *H. sacchari*. First, the Hewlett-Packard 5985-A spectrometer was used with a series of ion source temperatures and ionization voltages in the electron ionization mode. With the ion source temperature at 250°C and the ionization voltage at 70 eV, the mass spectra for the two preparations were essentially the same, except for minor peaks; these spectra were similar to the published spectrum for helminthosporoside (7). Next, the fragmentation conditions were altered to favor survival of higher mass ions: this was accomplished by using a lower ion source temperature (200°C) and a lower ionization voltage (35 eV). Under these conditions, the two preparations gave similar spectra, including the *m/e* 380 ion. However, Strobel's preparation had an ion at *m/e* 236 which was missing from all spectra of our preparations. This difference is important because *m/e* 236 was considered to be the molecular ion of the toxin (7).

Further comparisons of Strobel's preparation with ours were by high resolution peak matching, using the Varian CH-5 mass spectrometer. Ions resulting from fragments of the aglycone moiety of toxin were selected for examination, because that unit (the C-15 compound) was not included in the previously proposed stucture (7). Thus, the empirical formulae were determined for the ions at *m/e* 145, 157, 201, 217, and 380, which were evident in the spectra of both preparations. The predicted empirical formulas for these ions were identical for both preparations. The data indicate that our toxic molecule is the same as the one reported elsewhere, and that the proposed structure (7) must be revised.

DISCUSSION

Our highly purified toxin contained no detectable contaminants, as shown by thin layer chromatography and by gas chromatography of derivatives. Toxin purified by gas chromatography was hydrolyzed and the hydrolytic products were analyzed. The experiment confirmed that the products of hydrolysis (galactose and a 15-carbon compound) were derived from a single toxic molecule.

A preparation of helminthosporoside, kindly supplied by G. Strobel, was compared with our preparation of host-selective toxin from *H. sacchari*. The preparations had identical genotype specificity to sugar cane, identical behavior in all thin layer chromatography systems that we used, and very similar IR spectra. However, preparations described elsewhere were yellow (7); the sample we obtained from Strobel was yellow. Our highly purified toxin was colorless and our impure preparations were yellow. The mass spectra which we obtained from Strobel's and our preparations were very similar, with high mass ions at *m/e* 380. However, an ion at *m/e* 236 was present in Strobel's preparation but was missing in ours; the *m/e* 236 ion appears to be a contaminant. We conclude from the mass spectral data that the same toxic molecule was present in both preparations.

Many details seen in our NMR spectrum of the host-selective toxin are not evident in the published spectrum of helminthosporoside (7). The published spectrum (7) has a low signal to noise ratio, possibly resulting from low contamination with water, a low concentration of toxin, or other factors. The four distinct one-proton peaks in our spectrum at 4.95 to 5.41 ppm may have been lost in the signal noise in the published spectrum of helminthosporoside. The series of peaks at 1.35 to 1.2 ppm were interpreted to be from a proton in a cyclopropyl ring (7). In our experience, such peaks were evident at 1.19 to 1.04 ppm in spectra of impure toxin preparations (data not given); cleaner preparations retained selective toxicity but lacked these peaks. Limited data (not given) indicated that the 1.19–1.04 peaks were from peptide contaminants containing valine. A trace amount of the valine-containing peptide is suggested by a small peak at 1.08 ppm in the NMR spectrum of our preparation (Fig. 3).

Several kinds of data indicate that the true molecular weight of the toxin is >1000. The ion at *m/e* 380 (Fig. 2) probably does not represent the true molecular ion, because of limitations in the instrument and the method used. The *m/e* 380 ion, with the empirical formula $C_{21}H_{32}O_6$, is consistent with a structure containing one galactose and one 15-carbon unit. However, the mass spectrum for methylated toxin, obtained with the Varian CH-5 spectrometer with the EI/D method, contained an ion at *m/e* 1060; this indicates that toxin contains at least 5 galactose units plus a $C_{15}H_{21}$ moiety. The proton NMR data indicate 4–6 galactose units/toxin molecule. The ^{13}C NMR data, although not quantitative, also are consistent with 4–6 galactose units. The proton NMR of the Me_3Si derivative of toxin indicates 14–17 hydroxyls/toxin molecule, which is consistent with 5 galactose units. Results of the methylation analysis of galactose from hydrolyzed toxin suggest 5 galactose units in an unbranched chain. ^{13}C NMR spectra indicate that the oligosaccharide contains β, $1 \rightarrow 5$ linked galactose in the furanose form (5, 9).

An attempt was made to hydrolyze toxin with α and β-galactosidases (Sigma Chemical Co.). There was no liberation of galactose or C-15 compounds by these enzymes, used singly or in combinations (data not given).

Four different 15-carbon compounds were isolated after hydrolysis of the toxin by dilute acid. All the 15-carbon compounds had a molecular weight of 218, as determined by mass spectroscopy. These compounds appear to be convertible from one to the other. NMR data indicated that the compounds differ from each other in the positions of their double bonds and rings. The compounds may be sesquiterpenes, as indicated by the 15-carbon skeletons with double bonds. Furthermore, the molecules of some sesquiterpenes are known to be rearranged in dilute acid. The MS and hydrolysis data indicated that the 15-carbon unit is attached to the galactose chain by a galactosidic linkage. Acid hydrolysis of this linkage should give a 15-carbon unit bearing a hydroxyl group on the carbon that was involved in the galactosidic linkage. Further characterization of the 15-carbon moiety is underway.

In summary, the data discussed above show that toxin contains galactofuranose units linked by β, $1 \rightarrow 5$ bonds, plus a $C_{15}H_{21}$ moiety attached to the reducing end of the oligosaccharide. Several lines of evidence indicate five galactose units. The empirical formula of the aglycone unit indicates 5 points of unsaturation; spectral data indicate 3 double bonds and 2 rings (a sesquiterpene). Molecular weight of the toxin was calculated, tentatively, to be 1028.

Acknowledgments—We are grateful to Professors Charles Sweeley and W. H. Reusch for suggestions and help in interpretation of data, and to Dr. H. Nunez for interpretation of ^{13}C NMR data. We also thank Professors N. E. Good, C. J. Pollard, and K. Kohmoto for helpful comments and discussion.

Note Added in Proof—We recently became aware of work on *H. sacchari* toxin by R. C. Beier ((1980) Ph.D. thesis, Department of Chemistry, Montana State University). Beier suggests that the toxin may contain 2 galactose units and an aglycone ($C_{13}H_{22}O_2$).

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Additional references are found on p. 1710.

SUPPLEMENTARY MATERIAL

to

Isolation and Characterization of Host-Selective Toxin From Helminthosporium sacchari

by

Robert S. Livingston and Robert P. Scheffer

MATERIALS AND METHODS

Cultures. Isolates of Helminthosporium sacchari were obtained from Jack C. Comstock of the Hawaii Sugar Planters' Association, Gary A. Strobel of Montana State University, and Jack L. Dean of the U.S.D.A. Sugarcane Research Station at Canal Point, FL. Stock cultures of the isolates, which were similar in ability to produce toxin, were maintained on cane leaf agar (6). Cultures for toxin production were grown for 21 days at 21–23°C, in still culture in one liter 8oz bottles each containing 200 ml of Fries medium supplemented with 0.1% yeast extract (3). Toxin was present in culture fluids which were harvested by filtration through cheesecloth and Whatman No. 1 paper.

Toxin assay. Toxin was assayed by a standard procedure (4) based on induced leakage of electrolytes from susceptible but not from resistant tissue. All assays and all experiments were repeated two or more times. A toxin-sensitive (Co 453, and a toxin-insensitive clone (H60-7209 or H62-4610) were used in each assay. Plants were grown as described elsewhere (4).

Purification of toxin. Culture filtrate (5 liters) was concentrated by evaporation at reduced pressure to 500 ml. 20 g activated charcoal (Norit A from Sigma Chem Co.) was added to the concentrate, which was cooled to 4°C, stirred for 12 hours, and centrifuged at 10,000 × g for 8 minutes. The charcoal pellet containing bound toxin was re-suspended in 500 ml of 10% ethanol, stirred for 5 minutes, and centrifuged again. The supernatant was discarded. The pellet was resuspended in 25% ethanol (500 ml), stirred, re-centrifuged, and the supernatant again discarded. Toxin was eluted from Norit A by resuspending the pellet in 500 ml of 50% ethanol containing 1% ammonium hydroxide. To do this, the slurry was agitated for 10 minutes and centrifuged. This final elution step was repeated and the two 500 ml rinses were combined, filtered through Whatman glass fiber filter paper to remove traces of Norit A, and concentrated to 5 ml. Methanol (50 ml) was stirred slowly into the concentrated preparation and the solution was held at -15°C for 24 hr. A precipitate was discarded.

The methanol supernatant was concentrated to a syrup, raised to 3 ml with 50% methanol, and placed on a Sephadex LH-20 column (80 × 3 cm). The flow rate for this and all other columns was 0.05 - 0.1 ml/min. The column was developed with 50% methanol and 7 ml fractions were collected. Toxin was recovered in fractions from 427–469 ml. This step separated toxin from 3 related but non-toxic substances ("toxins") which will be discussed in results. Each fraction from the LH-20 (and other) column was assayed for biological activity and subjected to TLC to detect fractions containing toxin and toxins. The size and intensity of spots, developed on TLC plates sprayed with diphenylamine:aniline:phosphoric acid, indicated relative amounts of toxin and toxins. Only the fractions from the columns that were estimated to contain >80% toxin were processed further.

Toxin-containing fractions from the LH-20 column were combined, concentrated *in vacuo* and dissolved in one ml of water. Aliquots (0.5 ml) were placed on a Sephadex DAE anion exchange column (BS × 1.5 cm) and developed with water, 2 ml fractions were collected. The DAE Sephadex was swollen in 0.1 *N* NaCl and rinsed with water prior to pouring the column. The toxin was in fractions from 80–100 ml. Toxin-containing fractions were combined, taken to dryness, and dissolved in 1.0 ml methanol. A sample (25 mg dry wt) was streaked on each of several thin-layer plates (Merck silica gel-60, 20 × 20 cm, 0.25 mm thick) and developed to a 17 cm front with acetone:water (9:1). The toxin-containing zone was located by holding the plates in front of a fluorescent light immediately after removal from the solvent tank. Differential evaporation of the solvent resulted in a highlighting of the chromatographed toxin. The area was outlined quickly because it soon disappeared. To ensure the accuracy of this procedure a one cm strip of the TLC plate was removed and sprayed with indicator. The stained toxin always corresponded to the highlighted zone. If done carefully, this TLC procedure allowed almost complete separation of toxin from a similar non-toxic compound (toxin-1). The toxin-containing zone (R_f=0.5) was scraped from each plate and toxin was eluted from the gel with several rinses of methanol.

An aqueous solution (0.5 ml) of toxin from six thin-layer plates was chromatographed, with water as the solvent, on a column (0.9 cm) containing layers (from top to bottom) of Sephadex G-25, 2 cm, Sephadex SP cation exchange resin, 10 cm, Sephadex G-25, 2 cm, and Sephadex DAE anion exchange resin, 10 cm. Toxin was in the 10 to 14 ml portion of the eluate. These fractions were combined, concentrated, and dissolved in 0.3 ml of water. Next, the preparation was passed through a Biogel P-2 column (100–200 mesh, 115 × 1.35 cm), developed with water. Toxin was in the eluate at 126–138 ml. Fractions from 130–136 ml were combined and concentrated. The preparation caused leakage of electrolytes from sugarcane clone Co 453 at 10 mg/ml. Toxin was stable when stored in methanol at -15°C.

Preparation and use of toxin derivatives. A trimethylsilyl (Me$_3$Si) derivative of toxin was prepared by a standard procedure, for use in gas chromatography. Toxin in methanol was dried in a reaction vial at 30°C, using a jet of nitrogen. Trimethylsilylimidazole in dry pyridine (Tri-Sil 1, Pierce Chemical Co.) was added until the solution was diluted to 5 mg toxin/ml. Derivatization was complete after 5 minutes at 30°C. The Me$_3$Si-derivative was chromatographed on a 7 × 450 mm column of 3% Dexil 300 or 15 OV-1, using a Hewlitt-Packard 402 gas chromatograph equipped with a HP3380A peak integrator. The temperature was increased by 10°C per minute, from 170° to 300°C, and the helium flow rate was 60 ml/minute. Isothermal development was at 220°C, also with a helium flow rate of 60 ml/minute.

A trimethylsilyl (TMS) derivative of toxin was prepared for NMR determination of the number of hydroxyls on the toxin molecule. Tri-Sil-Z was used to derivatize toxin (10 mg), as described above. Thin-layer chromatography was used to separate TMS-toxin from the by-products and from reagents. The solution was streaked on a 20 × 20 cm plate and developed to a 17 cm front with chloroform:diethyl ether (3:1). The TMS-toxin was located with an indicator (1% vanillin in H_2SO_4) applied to a strip broken from the TLC plate, color was developed at 100°C for 5 min. Most of the by-products of the reaction had R_f values below 0.1. TMS-derivatized toxin (R_f 0.75) was removed and eluted with five rinses (3 ml each) of dry diethyl ether. The solution was filtered through a glass fiber filter and brought to dryness under reduced pressure. The residue was dissolved in 0.5 ml of deuterated chloroform.

Hydrolysis of toxin. Highly purified toxin was dissolved in aqueous 0.05 *N* trifluoroacetic acid in a 1.5 ml vial. Two volumes of chloroform were then added, the vial was sealed firmly with a Teflon-lined cap, and the preparation was brought to 95°C in a heating block. At 40 minute intervals the vial was cooled quickly, the chloroform phase was removed, a new aliquot of chloroform was added, and the vial was returned to the heating block. Total time of hydrolysis at 95°C was two hours, after which the aqueous phase was extracted three times, each time with one volume of chloroform, all chloroform extracts were combined and concentrated. The aqueous and the chloroform phases were analyzed for hydrolytic products.

Separation of chloroform-soluble products of toxin hydrolysis. The chloroform phase of hydrolyzed toxin was concentrated at reduced pressure, streaked on a TLC plate, and developed to a 17 cm front with chloroform:diethyl ether (1:1). Two spots were evident, one at R_f 0.15 and the other at 0.46. The two zones were removed from the plate, combined, and eluted with 5 rinses of chloroform. Combined rinses were filtered and concentrated to 0.3 ml. The sample was then placed on a LH-20 column (1.0 × 105 cm) and developed with dichloromethane:methanol:hexane (2:1:2). Each fraction (1.75 ml) from the column was subjected to gas chromatography. The fractions from 65 to 77 ml contained C-18a and b (C-18a peaked at 70 ml and C-18b at 72 ml), whereas fractions from 87 to 105 ml contained C-18c and d (C-18c peaked at 94 ml and C-18d peaked at 98 ml). Fractions containing peaks for each of the four products were re-chromatographed several times on the LH-20 column, to enrich the concentration of each. Highly enriched preparations of C-18c and C-18d were produced in this way, but C-18a and C-18b were not separated beyond a 60:40 ratio.

Methylation analysis of toxin. Toxin was methylated by Hakomori's method (1), as described by Jansson et al. (2). Dimethyl sulfoxide (Me_2SO) (0.3 ml) was added to dry toxin (0.5 mg) and the solution was sonicated for 5-10 min. A solution of dimsyl sodium (0.3 ml, was added under nitrogen, with continuous stirring. The solution was cooled on ice, iodomethane (1.5 ml) was added slowly, and the reaction mixture was incubated with continuous stirring for 2 hours at 22°C. Chloroform (5 ml) was added, and the solution was extracted three times with water (3-5 ml each time). The chloroform phase was concentrated under nitrogen. The residue was dissolved in acetone (0.4 ml) and chromatographed on an LH-20 column (0.9 x 25 cm), with acetone as the solvent, and 41 fractions were collected. Fractions containing methylated toxin were identified by spotting 5 µl aliquots on thin-layer plates which were developed with chloroform. Developed plates were sprayed with diphenylamine aniline:phosphoric acid and heated to 100°C to develop spots. Fractions 10 to 14, which contained methylated toxin, were combined and dried under nitrogen. The methylation procedure was repeated to insure complete methylation of toxin. Methylated toxin had no IR absorption at 3400 cm^{-1}, indicating no free hydroxyls.

Methylated toxin was hydrolyzed in 0.5M H_2SO_4 at 80° for 18 hours. Sulfate was removed by passing the solution through a small column (1 ml volume) of Dowex 11-8 (80-100 mesh) that had been converted to the acetate form. The hydrolysate was collected, and the column was rinsed with 2 ml of methanol. The eluate and rinses were combined, dried under nitrogen, and the partially methylated hexoses were reduced in an aqueous solution of $NaBH_4$ (10 mg/ml) at 22°C. The reaction was terminated after 2 hours by adding 2 drops of acetic acid. The solution was dried and the borate converted to its methyl ester for removal; this was accomplished by adding 2 ml of methanol and 2 drops of acetic acid. The vial was sealed and held at 100°C for 5 minutes, then evaporated under nitrogen. The esterification procedure was repeated two times. The final residue was dried and acetylated in 0.5 ml of acetic anhydride:pyridine (1:1 vol/vol) at 30°C for 1.5 hours. Toluene was added and evaporated under nitrogen, to remove excess reagents. The residue was dissolved in methylene chloride (1 ml) and extracted 3 times with water (0.5 ml each time). The organic phase was evaporated to remove all traces of water and dissolved in acetone or methylene chloride.

Gas chromatography-mass spectroscopy was used to identify each partially methylated alditol acetate. Retention times of the products on a column of 3% OV-225 (180 cm x 2 mm), developed isothermally at 170°, were compared with that of the standard, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylgalactitol (retention time, 3.13 minutes). Final confirmation of each structure was by comparison with published mass spectra (2). Quantitative determinations for each partially methylated alditol acetate product was determined by peak area integration with a Hewlett-Packard 3380A integrator.

RESULTS

Evidence of purity of the toxin preparation. Purified toxin was chromatographed on thin-layer plates, using several different solvent systems (Table 2) and several different indicator treatments. Toxin preparations gave a single distinct spot. The indicator reagents used to detect toxin on silica gel plates (No. 60 from E. Merck), and the color reactions with toxin, were as follows: concentrated sulfuric acid at 100°C for 10 min (black), iodine vapor (yellow), and Kemp's trichloride, in a saturated chloroform solution, heated (red), diphenylamine/aniline/phosphoric acid (2g/2 ml/10 ml, in 100 ml acetone) (green); and vanillin (1.0%) in H_2SO_4 (purple).

Table 2. Solvents used in thin-layer chromatography of toxin from Helminthosporium sacchari[a]

Solvent system	Proportions	R_f of toxin
Acetone:water	9:1	0.5
Acetone:water	19:1	0.15
Methanol:acetic acid:water	4:1:1	0.3
n-propanol:ethyl acetate:water	7:1:2	0.58
Ethyl ethyl ketone:water:methanol	17:4:7	0.14
Chloroform:methanol	4:1	0

[a]Chromatographed on Silica Gel 60, 0.25 mm hard-surfaced plates from E. Merck.

Gas chromatography of the Reyzl-derivative of the toxin gave a single peak. The peaks from several runs were collected in condensing tubes, from which the Reyzl-toxin was eluted with dry hexane. This highly purified derivative of toxin had the same retention time when rechromatographed by GC. It also had the same R_f (.75) on thin-layer plates developed with chloroform:diethyl ether, 3:1), as did the original derivatized preparation. This indicates no major structural alterations during gas chromatography.

A sample of the Reyzl-toxin purified by gas chromatography was combined with an equal volume of methanol. The solution was evaporated at 30°C with a jet of nitrogen, the volume was increased to 0.5 ml by the addition of aqueous 30% methanol. This procedure removed Reyzl-groups from the toxin, regenerating the original hydroxyls. When sufficient evaporation had occurred to remove all organic solvents, the volume was increased to 0.15 ml by adding trifluoroacetic acid, giving a 0.06 N final concentration of trifluoroacetic acid. This solution was heated for 15 minutes at 80°C, 0.3 ml of chloroform was added, and the standard procedure for toxin hydrolysis was followed. After hydrolysis, galactose was found in the aqueous phase and the other breakdown products of toxin were found in the chloroform phase, as shown by gas chromatography (see below). Thus, identical results were obtained with two procedures that have entirely different bases of derivatization (the standard procedure (Table 3) vs. gas chromatography). This is strong evidence that the hydrolytic products were derived from the toxin molecule and not from contaminants. Overall, the data confirm that the separation procedure gave a highly purified preparation of toxin.

Table 3. Chromatographic behavior of Helminthosporium sacchari toxin and related non-toxic substances (toxins). Preliminary processing included concentration of culture filtrates, followed by adsorption on Dowex A_1 eluted with 80% ethanol containing 1% ammonium hydroxide, and methanol precipitation.

	Eluate fractions containing toxin or toxin[b], or R_f values			
		Toxins		
Chromatographic System[a]	Toxin	I	II	III
LH-20 column, 7 ml fractions	61-67	68-71	85-93	108-112
DEAE column, 2 ml fractions	60-66	66-84	84-88	-
TLC, acetone:water (9:1)	R_f 0.5	R_f 0.56	R_f 0.65	R_f 0.7
TLC, acetone:water (19:1)c	R_f 0.15	R_f 0.3	R_f 0.5	R_f 0.6
SP-SAE column, 1 ml fractions	10-14	10-15	11-15	-
P-2 column, 2 ml fractions	64-69	69-73	-	-

[a]Descriptions of columns are given in the text.

[b]Thin-layer chromatography was used to identify toxin and toxins in each eluate fraction.

[c]The R_f values with this solvent varied, depending on wt of the sample and the presence of residual water.

Biological activity of the toxin preparation. The basal ends of excised sugarcane leaves were placed in small vials of toxin solution. Leaves from susceptible clones showed water congestion within 12 hours, followed by necrosis and drying. Leaves from highly resistant clones showed no toxic effects. Drops (1 or 5 µl) of toxin solution were placed on small puncture wounds near the basal ends of excised leaf sections, and the leaves were held in closed containers with high relative humidity (5). Highly sensitive leaves showed water congestion within 12 hours, "scorch lesions" (5) by 24 hours, and systemic effects by 48 hours after exposure to toxin. Leaves from highly resistant clones were not visibly affected. We have found that the visible reactions to toxin account be used as a quantitative assay (4).

The standard assay (4) was used to determine the minimum concentration of toxin that will cause loss of electrolytes from sensitive, intermediate, and highly insensitive clones of sugarcane. Highly purified toxin at 0.01 µg/ml caused leakage from leaf disks of clone Co 453, whereas 100 µg/ml had no effect on leaves of clone H32-8510. The values for intermediate clones fell between these extremes. Thus, the most insensitive clone tolerated at least 10,000 times higher concentration of toxin than did the highly sensitive clone.

General characteristics of toxin. Highly purified toxin dried over P_2O_5 under vacuum was colorless and glass-like. When exposed to moisture in the air, the preparation turned to a colorless syrup, indicating that toxin is very hygroscopic. When the colorless syrup was re-dried, the appearance and the biological activity were the same as for the original preparation.

Purified toxin was very soluble in water and methanol; it was slightly less soluble in dimethyl sulfoxide, pyridine, ethanol, and butanol. Toxin was sparingly soluble in acetone, and was insoluble in chloroform, ethyl acetate, and several other nonpolar solvents. Toxin did not reduce Benedict's reagent for reducing sugars, and gave a negative ninhydrin reaction.

We were not successful in eluting non-derivatized toxin from gas chromatography columns. The Reyzl-derivative of intact toxin required a temperature of 320°C to be eluted, indicating that toxin probably is a relatively large, non-volatile compound.

Toxin solutions did not absorb light in the UV range above 210 nm, nor in the visible range. The IR spectrum showed absorption at 1640 cm^{-1}. Raman spectroscopy of toxin also gave strong absorption at 1640 cm^{-1}. These data indicate the presence of non-conjugated double bonds.

An estimate of the amount of galactose in the toxin molecule. Toxin (0.5 mg) was hydrolyzed by the standard procedure and the aqueous phase was dried at 30°C with a jet of nitrogen; the residue was rinsed several times with methanol and dichloromethane to remove traces of trifluoroacetic acid. The residue was dissolved in water and an aliquot containing the aqueous phase from 25 µg of toxin was dried in a reaction vial and rinsed with dichloromethane to remove traces of water. Tri-Sil-Z (50 µl) was added and the vial was held at 40°C for 10 minutes to insure complete derivatization. A standard was prepared by derivatizing galactose (0.5 µg/ml) with Tri-Sil-Z, which forms the penta-Reyzl-derivative. Aliquots (5 µl) of the toxin products and the standard were injected into the GC column (2% OV-1, 1.8 meters long, and developed isothermally at 170°C. Peak areas were integrated. The results indicated that hydrolysis of 1.0 µg of toxin released 0.7 µg of galactose.

Methylation analysis of toxin. GPD and hydrolysis data indicated more than one galactose moiety per molecule of toxin. Methylation analysis was used to determine the substitution pattern of these galactose units in toxin. The procedure gave two products, with retention times on the OV-225 column of 3.02 and 5.95 minutes. The identities of the products were then determined by comparison of their mass spectra with published spectra (2). The presence of several unique ions in each spectrum confirmed the presence of the two partially methylated alditol acetates [(1,4-di-O-acetyl-2,3,5,6-tetra-O-methylgalactitol (m/e 99, 87, 205) and 1,4,6-tri-O-acetyl-2,3,5-tri-O-methylgalactitol (m/e 99, 113, 233)]. The ratios of these two compounds varied with the concentration of acid used in hydrolysis. This may be due to the instability of the galactose which is in the furanose form. The lowest concentration of H_2SO_4 that completely hydrolyzed the methylated toxin and caused minimum destruction of products was 0.5 N.

Mole percent composition for the two partially methylated alditol acetates was determined by integration of the peak areas in the gas chromatogram. The average molar ratio of 1,4-di-O-acetyl-2,3,5,6-tetra-O-methylgalactitol to 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol was 1:4, although ratios of 1:3 and 1:5 were obtained. This, along with GPD and hydrolysis data, suggests an oligosaccharide with 5 galactose units in the toxin molecule. The methylation data are compatible with either of two different linkage patterns for the oligosaccharide: (a) a terminal galactoforanose with internal 1→4 linked galactopyranose units (ruled out by ^{13}C-NMR data), or (b) all galactoforanose units with 1→5 linkages.

Non-toxic compounds associated with toxin. Toxin eluates from Dowex A were chromatographed on an LH-20 column, as described in the toxin purification procedure. A 5 µl sample of each 7 ml eluate fraction was placed on a thin-layer plate and developed with acetone:water (19:1). The plates were then sprayed with diphenylamine:aniline:phosphoric acid and heated. This procedure showed that four components (with different R_f values on the TLC plates) were eluted from the LH-20 column in different fractions. Bioassays showed the compound eluted first (fractions 61-67, Table 3) was the toxin, compounds eluted in later fractions caused no toxic symptoms in sugarcane leaves, and caused no leakage of electrolytes from leaf disks. For convenience the non-toxic components are called toxins I, II, and III. The elution volumes and R_f values for toxins in several chromatography systems are given in Table 3. Toxins I and II were purified from culture filtrates by the usual procedures. Toxin III was highly soluble in methanol and sparingly soluble in water and chloroform. Therefore, toxin III was eluted from the ion-exchange columns with 50% methanol and from the P-2 column with methanol. Acetone:water (19:1) was used as the solvent in thin-layer chromatography of toxins II and III.

The isolated toxins were hydrolyzed and the hydrolytic products examined by the procedures described for the toxin. Galactose was the only product found in the aqueous phase. The chloroform phases from hydrolyzed toxins I and II were analyzed by GC-MS; four major peaks were obtained. The retention times and mass spectra of these hydrolytic products of toxins were identical to those for the four 15-carbon hydrolytic products of toxin (C-15a, b, c and d) (Fig. 5). The toxin and toxins I and II differed in the relative amounts of each of the four different C-15 products of hydrolysis. The chloroform phase of hydrolyzed toxin III contained C-15c and d, with C-15a and b in trace amounts, a new 15-carbon product, unique to toxin III, was present in high concentrations. It had a retention time between those of C-15a and b.

The mass spectra of intact toxin and the toxins differed only in relative abundances of a few of the ions; the same basic fragmentation patterns were evident when both were monitored up to m/e 600. The protein GPD of toxin and toxins I and II contained the same peaks. The two toxins differed in the area of the peaks attributed to the proteins or galactose, suggesting that toxin and the two toxins differed from each other primarily in the number of galactose units present. An GPD spectrum was not obtained for toxin III.

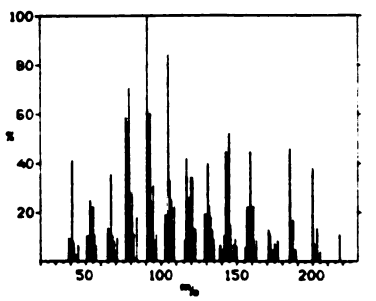

Fig. 5. Mass spectrum of one of the chloroform-soluble hydrolytic products (C-15d) of toxin from Helminthosporium sacchari. Sample was introduced into the source (temp. 280°C) by gas chromatography and ionized by electron ionization (70 ev). The gas chromatography conditions are described in the text. Mass spectra of these compounds (C-15a, b, c, and d) obtained by chemical ionization with methane confirmed the molecular ions as m/e 218; high resolution mass metering gave the empirical formula as $C_{15}H_{21}O_4^+$.

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EXPERIMENTAL II

CONVERSION OF *HELMINTHOSPORIUM SACCHARI* TOXIN TO TOXOIDS BY β-GALACTOFURANOSIDASE FROM *HELMINTHOSPORIUM*

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Conversion of *Helminthosporium sacchari* Toxin to Toxoids by β-Galactofuranosidase from *Helminthosporium*[1]

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ABSTRACT

Helminthosporium sacchari **produces a host-selective toxin and structurally related nontoxic compounds, here referred to as 'toxoids.' Toxin and the three toxoids were each isolated to a high level of purity and were hydrolyzed under acidic conditions. The released galactose was measured by a galactose oxidase/peroxidase assay. Toxin was found to contain four units of galactose per molecule, as previously reported. Toxoids I, II, and III contained one, two, and three units of galactose, respectively. In cultures of the fungus, toxin concentration peaked at 3 weeks, followed by a rapid decline; as toxin levels fell, the total amount of toxoids increased. An enzyme with β-galactofuranosidase activity was found in small amounts in the cultures of *H. sacchari*; the enzyme converted toxin to the toxoids *in vitro*. β-Galactofuranosidase was previously known from very few microorganisms; therefore, several pathogenic *Helminthosporia* and other fungi were tested for production. β-Galactofuranosidase activity in culture filtrates and mycelia of *H. victoriae*, *H. maydis*, *H. carbonum*, and *H. turcicum* was much greater than in filtrates and mycelium of *H. sacchari*. More work is needed to determine the significance of enzyme production by these fungi. No β-galactofuranosidase was evident from *Fusarium oxysporum* and *Cladosporium cucumerinum*.**

Helminthosporium sacchari (Van Breda deHaan) Butler produces in culture a host-selective toxin (11). Clones of sugarcane which are highly sensitive to the toxin are also susceptible to the pathogen, whereas clones that are insensitive to toxin are resistant to the pathogen (10). The toxin was first characterized as 2-hydroxycyclopropyl-α-D-galactopyranoside, or 'helminthosporoside' (12). Our data indicate that this structure is incorrect, and that the toxin molecule contains four to six β-galactofuranose units plus a sesquiterpene (3, 4). Macko *et al.* (7) have proposed a structure which contains four galactose units attached to an aglycone ($C_{15}H_{24}O_2$). Several nontoxic compounds similar to toxin have been found in culture filtrates, along with toxin (3, 4). Pretreatment of sensitive sugarcane tissues with the related compounds (toxoids) reduced toxin-induced electrolyte leakage (5). The toxoids were purified and were all found to contain an aglycone with the same mol wt as the sesquiterpene that is part of the toxin molecule. Thus, the toxoids differ from toxin only in the number of galactose units in the molecule (5).

Early observations indicated that toxin in cultures of *H. sacchari* reaches a peak in 3 weeks, followed by a rapid decline. The decline in toxin could be caused by a β-galactofuranosidase such as that described by Rietschel-Berst *et al.* from *Penicillium charlesii* (9). We have found that the *Penicillium* enzyme will release galactose from HS toxin[2] and from toxoids, thus confirming the β-galactofuranose conformation of these compounds. We report here the isolation of an enzyme having β-galactofuranosidase activity from cultures of several species of *Helminthosporium*. The enzyme may be responsible for the rapid drop in toxin concentration in culture fluids of *H. sacchari* and the concurrent increase in toxoids. An abstract of some of this work was published (6).

The term 'toxoid' is rational and convenient for the nontoxic compounds related to HS toxin. This follows previous use of toxoid for inactive forms of toxins involved in animal diseases. However, the toxin and toxoids from *H. sacchari* are not known to be antigenic, in contrast to toxins and toxoids from animal pathogens. Three toxoids from *H. sacchari* are designated I, II, and III, in reference to the numbers of galactose units in the molecules, but with no reference to isomers. These same three toxoids were called 'noxins' III, II, and I, respectively, in an earlier report (4).

MATERIALS AND METHODS

Helminthosporium sacchari was grown for toxin, toxoid, and enzyme production in still culture at 21 to 23°C, in 1-L Roux bottles each containing 200 ml of Fries medium supplemented with 0.1% yeast extract (8). In time course studies, the culture fluids from three bottles were harvested each week and filtered through Miracloth. In toxin studies, the bottles and fungal mats were rinsed with 25 ml of 50% methanol; the rinse solution was added to the culture filtrate which was then concentrated under reduced pressure (at 37°C) to one-tenth the original filtrate volume. Norit A (2.5 gm) was added, the concentrate was stirred at 4°C for 15 h, and the toxin and toxoids were extracted as previously described (4). To recover all of the toxoid with lowest mol wt, a final dichloromethane:methanol (1:1) extraction of the Norit A was required. The solutions were combined, filtered, and concentrated to a syrup as described above. Methanol (50 ml) was added slowly, the solution was held at −15°C overnight, and the resultant precipitate discarded. The supernatant solution was concentrated and made to 3.0 ml with methanol. This preparation, designated 'A,' was used for GLC determinations of toxin and toxoids. Highly purified preparations (designated 'B') of toxin and toxoids were obtained by the procedures described previously (4). A trimethylsilyl derivative of the preparations was subjected to GC to verify the absence of free galactose or other compounds. Toxin and toxoids were stable when stored in methanol at −15°C. Dry weights of the preparations were determined after drying at 110°C.

An aliquot (10–20 μl) of preparation A was dried and Me_3Si derivatives (50–100 μl, total volume) of the toxin and the toxoids

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[2] Abbreviations: HS toxin, the host-selective toxin from *H. sacchari*; Me_3Si, trimethylsilyl.

prepared as previously described (4). The Me_3Si derivatives (2–6 μl/injection) were chromatographed on a 2 × 450 mm column of 2% OV-1 in a Varian 3700 gas chromatograph with a flame ionization detector. Temperature was increased by 30°C/min from 130°C to 350°C, followed by an isothermal run at 350°C for 5 min. The recorded peaks were cut out, weighed, and quantified by comparison with standard curves for purified toxin and toxoids. Toxin was also measured by an electrolyte leakage bioassay (10).

To determine the amount of galactose present in the toxin and toxoid molecules, aliquots of purified preparations (B) were hydrolyzed in 0.5 M TFA at 95°C for 2 h in a sealed vial. The acid was removed from the opened vial at 40°C, using a jet of N_2. The residue was dissolved in 0.2 M phosphate buffer (0.5 ml, pH 7.0). Galactose released from toxin and toxoids was measured by a modified version of the procedure of Fischer and Zapf (2). The assay solution was adjusted to 0.5 ml with 100 mM phosphate buffer (pH 7.0). A solution (200 μl) which contained peroxidase (5 units) and *o*-cresol (3.8 mmol) was added, followed by 50 μl of galactose oxidase solution (1.25 units). The reaction was allowed to proceed at 37°C for 30 min, when the absorption at 410 nm was measured. Peroxidase (type II) and galactose oxidase (type V) were obtained from Sigma Chemical Co.

Fungal cultures for enzyme production were grown as described above. Cultures were harvested and the fungal mat was squeezed to remove excess liquid. The solution was filtered and precipitated with ammonium sulfate (476 mg/ml) at 4°C. A pellet was collected by centrifugation at 20.000*g* for 20 min; the pellet was resuspended in 2.0 mM phosphate buffer at pH 7.0. Enzyme in the mycelium was extracted in a grinding medium containing phosphate buffer (50 mM, pH 7.0) and 2-mercaptoethanol (10 mM). The mycelium was disrupted with a Sorvall Omni-mixer run at high speed at 4°C for 4 min. The resulting slurry was centrifuged at 20.000*g* for 15 min, and the supernatant solution was precipitated with ammonium sulfate (476 mg/ml) at 4°C. A pellet was collected and resuspended as described above. This preparation was used when comparing enzyme activities of various fungal species and for weekly determinations of enzyme activities.

Another procedure was used to obtain a more purified enzyme for GLC studies of enzyme hydrolysis products of toxin. The pellet from the ammonium sulfate precipitation was suspended in water, dialyzed against phosphate buffer (10 mM, pH 7.0), and centrifuged (20.000*g* for 15 min) to remove insoluble materials. An aliquot of the dialyzed solution, containing 10 mg of protein, was applied to an upward-flowing Bio-Gel P-150 column (2.6 × 31 cm), which was developed with phosphate buffer (10 mM, pH 7.0). Enzyme activity was eluted as a peak in fractions from 58 to 74 ml; enzyme elution came immediately after void volume, indicating a mol wt >150,000. The active fractions were combined and dialyzed against phosphate buffer (1.0 mM, pH 7.0). The dialyzed solution was then concentrated under reduced pressure at 25°C to one-fourth of the original volume and the pH was adjusted to 7.0. Aliquots were frozen for storage.

The enzyme was assayed using HS toxin as the substrate. Toxin was purified as previously described (4), omitting the TLC and final gel column steps. The preparation was free of galactose but contained a trace of toxoids. Enzyme activity was determined at 37°C, using 50 or 100 μl of a solution containing acetate buffer (20 mM, pH 4.5); the preparation was incubated for various times, up to 20 h. The amount of free galactose was determined by the galactose oxidase assay (2), as described above. One unit of enzyme activity was defined as the amount of enzyme required to release 1.0 μg galactose/h from 1.0 mg of toxin in a volume of 50 μl at 37°C. When possible, all assays were run at enzyme concentrations able to release 20.0 μg galactose in 6 h. Specific enzyme activity is presented as activity units per μg of protein. The galactose in toxin and toxoids is in the furanose form (3, 4) and therefore is not a substrate for galactose oxidase. Protein was determined by the procedure of Bradford (1).

All experiments were repeated one or more times.

RESULTS

Determination of Galactose in Toxin and Toxoid Molecules. The mol wt for the toxin and the three toxoids (I, II, and III) were calculated to be 884, 398, 560, and 722, respectively. These weights are calculated on the assumption that the molecules contain the aglycone ($C_{15}H_{24}O_2$) (7) plus 1 or more units of galactose.

Purified samples (50 μg each, preparation B) of toxin and the three toxoids were individually hydrolyzed and the amounts of galactose released were determined by the galactose oxidase/peroxidase assay. Hydrolysis of 50 μg toxin should release 40.7 μg galactose; the 40.9 μg obtained experimentally (Table I) confirms the assignment of 4 units of galactose/toxin molecule. Toxoid III released 36.4 μg galactose (theoretical, 37.4), indicating that the compound contained 3 units of galactose. Toxoids II and I released 33.4 and 21.9 μg galactose (theoretical, 32.1 and 22.6); this confirms the assignment of 2 and 1 units of galactose, respectively.

Accumulation of Toxin and Toxoids in Culture Fluids of *H. sacchari*. Toxin production in culture was shown by electrolyte leakage assays (10) to decline after reaching a peak at 3 weeks (data not shown). We then reexamined the time course of toxin production in culture, using quantitative GLC of Me_3Si derivatives of the toxin and toxoids. The GLC data showed clearly that toxin concentration peaked in 3-week-old cultures, and that toxin titers declined for the next 3 weeks (Fig. 1). From 3 to 4 weeks, the total

Table I. *Galactose Released by Acid Hydrolysis of Purified Toxin and Toxoids (50 μg each)*

Galactose was quantified by a galactose oxidase/peroxidase assay.

	Galactose Recovered			
	Toxin	Toxoid III	Toxoid II	Toxoid I
	μg			
Experimental value[a]	40.9 ± 0.9	36.4 ± 1.0	33.4 ± 0.7	21.9 ± 0.7
Theoretical value[b]	40.7	37.4	32.1	22.6

[a] Mean value and SD of four replicates.

[b] These values apply if the molecules of toxin, toxoid III, toxoid II, and toxoid I contain 4, 3, 2, and 1 units of galactose, respectively.

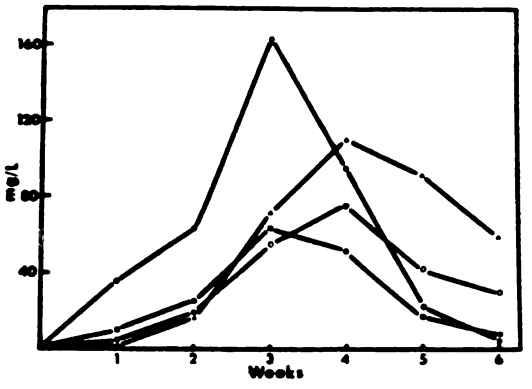

FIG. 1. Concentrations of toxin (●), toxoid III (■), toxoid II (○), and toxoid I (▲) in culture filtrates of *H. sacchari*, as determined by quantitative GLC.

toxoid levels increased as the toxin titer fell. The results suggest that the cultures may contain an enzyme capable of cleaving the β-1,5-lined galactofuranose units from HS toxin, thus producing toxoids.

Detection of β-Galactofuranosidase Activity in Cultures of *H. sacchari*. The rapid drop in toxin titer from the 3rd to the 5th week in culture suggested that maximum activity of a hypothetical enzyme should be found in the culture filtrate during this period. Filtrates from cultures that were 2 to 6 weeks old were dialyzed against water, then against 25 mM acetate buffer (pH 4.5). The volume was maintained at that of the original filtrate. Toxin (1 mg) was added to a 100-μl sample of the dialyzed enzyme preparation; no galactose was released during incubation for 15 h at 24°C. Therefore, a more concentrated solution of the enzyme was prepared from culture filtrates. The proteins from 3-week-old cultures were obtained as described in "Materials and Methods," the ammonium sulfate precipitated pellet was collected by centrifugation, resuspended in water, and dialyzed. Half the preparation was brought to pH 4.5 with acetate buffer and the other half to pH 7.0 with phosphate buffer. The final volume was one-fortieth of the original filtrate volume. Toxin (0.5 mg/50 μl of enzyme solution) was added as substrate and the mixture was incubated at 24°C for 20 h. The reaction was stopped by adding eight volumes of methanol. A precipitate was removed by centrifugation and a portion of the solution chromatographed on thin-layer silica plates with acetone:water (9:1). Toxin, toxoids, and galactose were made visible by spraying the plates with an indicator (diphenylamine, 2 g; aniline, 2 ml; phosphoric acid, 10 ml; acetone, 90 ml) and heating to 100°C.

The enzyme preparation at pH 4.5 cleaved 1 unit of galactose from approximately 25% of the toxin molecules, producing toxoid III. A trace of toxoid II was detected (produced by the removal of 1 galactose unit from toxoid III); no toxoid I was detected. When the reaction mixture was incubated at 24°C (pH 7.0) or at 0°C (pH 4.5 and 7.0), only a trace of galactose was released and a trace of toxoid III was detected after 20 h. The enzyme preparation was held at 100°C for 20 min, then was incubated with toxin at 24°C for 20 h; again, only traces of galactose and toxoid were detected.

The data show that the culture filtrate contains small amounts of an enzyme capable of hydrolyzing the β-1,5-galactofuranoside linkage in HS toxin and in the toxoids. The enzyme had maximum activity below pH 5.0; activity dropped rapidly at pH levels above 5.0. The pH of culture fluids was 3.5 at 21 d and increased to pH 5.0 at 32 d. Thus, conditions in culture favored enzyme activity, yet there was insufficient activity in the culture fluids to account for the rapid decline in toxin concentration which occurred from 21 to 32 d (Fig. 1).

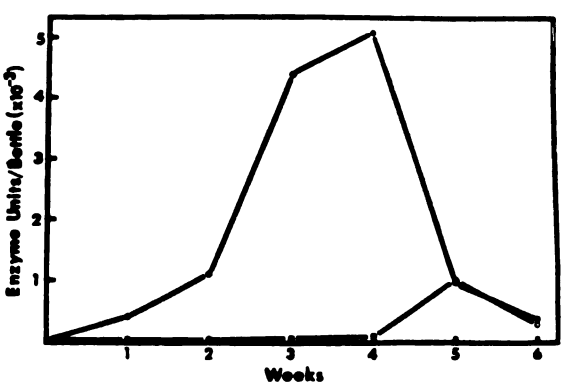

FIG. 2. β-Galactofuranosidase activities in culture fluids (○) and mycelium (●) of *H. sacchari* over a 6-week period. Cultures were grown in Roux bottles, each containing 200 ml of medium.

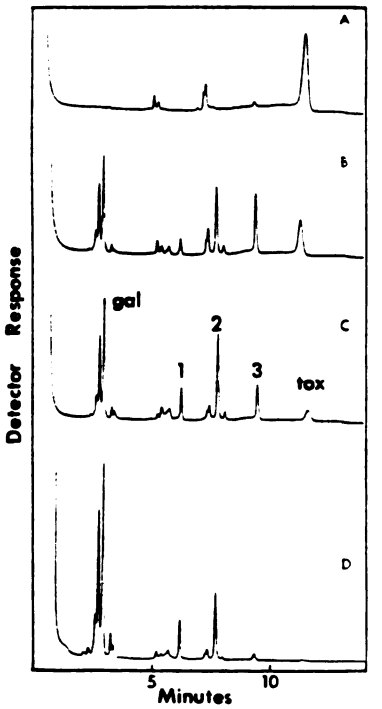

FIG. 3. GLC of products resulting from enzyme hydrolysis of HS toxin. Products are galactose (gal) and toxoids 1, II, and III (1, 2, and 3, respectively). Determinations were made with trimethylsilyl derivatives, using a temperature regime of 130°C for 1.0 min followed by increases of 30°C/min up to 350°C, which was held for 5.0 min. A, Substrate control; B, products obtained when the enzyme preparation was diluted 1:4; C, products when enzyme preparation was diluted 1:2; D, products when enzyme preparation was not diluted.

The amount of β-galactofuranosidase in the fungal mat and in culture filtrate was then determined at weekly intervals for 6 weeks (Fig. 2). Enzyme activity in the culture filtrate was barely detectable during the first 4 weeks: values ranged from 10 to 100 units/bottle. By week 5, the pH of the filtrate was above 6.0 and most of the toxin was gone. The amount of enzyme in the culture solution remained below the level that would be required to convert toxin to toxoids at the observed rate. There was substantially more enzyme activity in the mycelium than in the culture fluids; activity was detected in 1-week-old cultures, and reached a peak at 4 weeks (Fig. 2). However, we were not able to determine the amount of β-galactofuranosidase activity in the intact mycelium; therefore, we could not determine losses during preparation of enzyme.

Conversion of Toxin to Toxoids *In Vitro* by β-Galactofuranosidase. HS toxin was hydrolyzed with a highly purified preparation of β-galactofuranosidase which was kindly provided by Dr. J. E. Gander. At pH 4.6, the enzyme cleaved galactose from toxin, producing all the toxoids.

The enzyme was prepared from the mycelium of 4-week-old cultures of *H. sacchari*, using the method which included chromatography on a Bio-Gel P-150 column (see "Materials and Methods"). Several dilutions of the enzyme preparation were allowed to react with toxin (1.0 mg) in 100 μl acetate buffer (200 mM, pH 4.6) at 37°C for 18 h. The reaction was stopped by adding

Table II. *β-Galactofuranosidase Activity in Culture Filtrates and Fungal Mats of Helminthosporium and Other Species*

The fungi were grown in still culture (20 ml modified Fries solution in 125-ml flasks) for 23 d at 22°C.

Fungus	Isolate No.	Culture Filtrates		Mycelial Mat	
		Units/flask	Specific activity	Units/flask	Specific activity[a]
H. maydis race T	1	9,200	3.1	3,200	3.2
H. maydis race T	2	13,600	3.2	5,300	4.5
H. maydis race T	3	3,000	1.0	2,500	2.0
H. carbonum race 3		5,800	5.0	18,000	11.8
H. carbonum race 1		1,920	5.0	12,750	5.8
H. turcicum race 2		12,000	5.5	11,750	6.4
H. victoriae		24,000	1.8	12,250	4.8
H. sacchari[b]	1	80	0.08	1,800	0.45
H. sacchari	2	180	0.05	775	0.65
H. sacchari	3	40	0.05	775	0.24
H. sacchari	4	160	0.05	425	0.63
H. sacchari	5	20	0.05	1,550	0.35
H. sacchari	6	60	0.07	1,250	0.39
H. sacchari	7	60	0.09	1,425	0.27
H. sacchari	8	60	0.05	1,550	0.71
H. sacchari	9	40	0.05	1,400	0.30
H. sacchari	10	20	0.05	1,600	0.55
F. oxysporum		ND[c]		ND	
C. cucumerinum		ND		ND	

[a] Enzyme units/μl divided by the μg protein/μl of the enzyme preparation.

[b] Isolates of *H. sacchari* are from Florida (1–6), Hawaii (7–8), and Australia (9–10).

[c] None detected.

0.9 ml of methanol and the precipitate was removed by centrifugation. An aliquot of the solution was dried, Me_3Si derivatives were prepared, and the derivatized products were separated by GLC. The highest concentration of enzyme converted all the toxin to toxoids and galactose within 18 h (Fig. 3); most of toxoid III also was hydrolyzed. Less toxin was hydrolyzed by lower concentrations of the enzyme. There was no evidence of galactose dimers in the reaction solutions, indicating that the enzyme cleaves only the terminal unit of galactose, as does the enzyme described by Rietschel-Berst *et al.* (9). Toxoid I did not increase to high concentrations under these reaction conditions, although it does in the culture filtrates. The small peaks adjacent to the large galactose peaks were identified, by comparison with standards, as two sesquiterpenes which were found previously as acid hydrolysis products of toxin and toxoids (4). This indicates that some of the toxin was completely hydrolyed, liberating galactose and the sesquiterpenoid core of the toxin.

Production of β-Galactofuranosidase by Several *Helminthosporium* and Other Species. Large amounts of β-galactofuranosidase have been isolated from culture filtrates of *Penicillium charlesii* (9), in contrast to the low activity found in filtrates of *H. sacchari*. Accordingly, several *Helminthosporia* and other fungi were tested for β-galactofuranosidase activity (Table II). The fungi were grown in stationary culture for 23 d in 125-ml Erlenmeyer flasks, each containing 20 ml of modified Fries medium. Enzyme solutions were prepared from filtrates and from fungal mats. Assays showed that pH 4 to 5 was optimum for activity of the enzymes from each species.

The levels of β-galactofuranosidase activity in the culture filtrates of each of four species of *Helminthosporium* (*H. maydis*, *H. carbonum*, *H. victoriae*, and *H. turcicum*) were much greater (11–680 times) than the activity in the culture filtrate of *H. sacchari* (Table II). Fungal growth and protein levels in cultures of the several species were comparable; each isolate gave reproducible results. The amount of β-galactofuranosidase activity in the mycelium of *H. sacchari* was in all cases less than the amounts in the other *Helminthosporium* species. There was no detectable β-galactofuranosidase activity in the mycelium or culture filtrates of *F. oxysporum* or *C. cucumerinum*.

DISCUSSION

We reported previously that a host-selective toxin and three different toxoids, all containing galactose and a sesquiterpene, are produced by *H. sacchari* (3, 4). However, we did not determine the exact number of galactose units in the molecules. Mass spectral, NMR, and other data indicated that the toxin and toxoid molecules contained an aglycone, and that the aglycone from toxin and toxoids had the same mol wt. We also found that galactose in the toxin molecule is in the furanose form and is linked by β-1,5 bonds. We now report that there are 4, 3, 2, and 1 units of galactose in HS toxin, toxoid III, toxoid II, and toxoid I, respectively. These values were determined by accurate measurement of the galactose released by acid hydrolysis of toxin and toxoids. The results for toxin confirm the report of Macko *et al.* (7). Each of the toxoids may exist in three different isomers, as indicated by Macko *et al.* for toxin (7). A consideration of the isomers is outside the scope of this report.

GLC data showed a rapid drop in toxin concentration in *H. sacchari* cultures from week 3 to week 5. Electrolyte leakage assay also showed that toxin levels peaked at 21 d, and declined to nondetectable levels at 42 d. Sucrose in the medium was depleted by 15 d, and fungal growth stopped by 18 d (data not given). As toxin concentrations dropped, there was an increase in the amount of toxoids in the filtrates. The toxoids could result from enzymic or other breakdown of toxin, but there are other possibilities. Toxin and each of the toxoids could be synthetic end-products, or toxoid synthesis and enzymic breakdown of the toxin could occur concurrently. Cultures always contained toxoids when toxin was present, suggesting that toxin and toxoids could be synthetic end-products.

Toxin was shown to be converted to toxoids by a β-galactofuranosidase that was detected in cultures of *H. sacchari*. However, no enzyme activity was detected when dialyzed preparations of the culture fluids were added to purified toxin at concentrations up to 100-fold greater than that originally present in culture fluids. Thus, there was not enough enzyme activity in culture fluids to convert toxin to toxoids at the observed rates. β-Galactofuranosidase activity was detected when proteins in culture filtrates were precipitated with ammonium sulfate and dissolved in a small volume of water. Conditions for detection included concentrations of enzyme and toxin that were 4,000-fold greater than those found in filtrates of 3- to 5-week-old cultures. Under these conditions, only one-fourth of the toxin was cleaved; thus, the enzyme preparation from the culture fluids had <0.1% of the β-galactofuranosidase activity required to hydrolyze toxin at the rate that it disappears from cultures.

If β-galactofuranosidase activity leads to loss of toxin from cultures, then the enzyme must be associated with the mycelium. When toxin loss from culture fluids was most rapid, 98% of the β-galactofuranosidase activity was in the mycelium and only 2% was in the fluids. Even the activity in the mycelium may not be sufficient to account for a conversion of toxin to toxoids at the observed rate; thus, other mechanisms may be involved. Perhaps toxin or toxoid are brought together with the enzyme at particular locations in the cell. Another possibility is that the enzyme is attached to the cell wall and can convert toxin to toxoids without movement through the plasma membrane; this has not been examined. Finally, we were not able to determine the amount of

β-galactofuranosidase activity lost during the isolation of the enzyme from the mycelium; this could account for the shortage of enzyme needed to convert toxin to toxoid.

β-Galactofuranosidase has been reported from very few microorganisms; therefore, we examined several plant pathogens for ability to produce the enzyme. Isolates of *H. maydis*, *H. carbonum*, *H. turcicum*, and *H. victoriae* accumulated 11- to 680-fold more β-galactofuranosidase activity than did any one of the 10 isolates of *H. sacchari*. The enzyme activities in both the mycelium and the culture fluids of all tested isolates of *H. sacchari* were far less than the activities of the other *Helminthosporium* species. In contrast, cultures of *Cladosporium cucumerinum* and *Fusarium oxysporum* contained no detectable β-galactofuranosidase activity, indicating that the enzyme is not ubiquitous among fungi. Further work is necessary to determine the significance of high enzyme production by some species, low production by others, and no production by still others. The data do not prove that HS toxin is absent from cultures of some *Helminthosporia* because enzyme levels are high.

It seems likely that the activity of β-galactofuranosidase in young cultures of *H. sacchari* is low enough to allow HS toxin to accumulate. The enzyme probably contributes to the disappearance of toxin in mature cultures. The possible production and significance of β-galactofuranosidase in diseased tissue remains to be determined.

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EXPERIMENTAL III

TOXIC AND PROTECTIVE EFFECTS OF ANALOGS OF *HELMINTHOSPORIUM SACCHARI* TOXIN ON SUGARCANE TISSUES

TOXIC AND PROTECTIVE EFFECTS OF ANALOGS OF *HELMINTHOSPORIUM SACCHARI* TOXIN ON SUGARCANE TISSUES

Abstract

Host-selective toxin and three groups of smaller, structurally related compounds were isolated from culture fluids of *Helminthosporium sacchari*. The lower mol wt compounds (toxoids) differ from HS toxin in the number of galactose units per molecule. Toxoids protect sugarcane tissues from toxin, as shown by use of the assay based on toxin-induced loss of electrolytes. Toxoid III (3 units of galactose/molecule) was most effective at preventing toxicity; it gave 90% protection at a 24:1 ratio, on a molar basis. Toxoid II (2 units of galactose) was intermediate, and toxoid I (1 unit of galactose) provided the least protection against HS toxin. Protection was apparent when tissues were exposed to toxoid III for one h, rinsed to remove free toxoid, then exposed to toxin. The data suggest that toxoids act as competitive inhibitors of HS toxin. This is consistent with the hypothesis that toxin binds to a receptor molecule in susceptible cells. Toxoids I and II induced no loss of electrolytes from any of the tested clones of sugarcane. Toxoid III caused losses and runner lesions on clones NG 77-234 and NG 77-82, but not on clones Co 453 and NG 77-103; all four clones are equally susceptible to *H. sacchari*.

INTRODUCTION

Helminthosporium sacchari (Van Breda deHaan) Butler, the cause of eyespot disease of sugarcane, produces in culture a host-selective toxin (HS toxin) (13) and several structurally related compounds (3,4,7). Clones of sugarcane that are highly susceptible to the pathogen are sensitive to HS toxin and clones that are highly resistant to the pathogen are insensitive to the toxin. HS toxin, which was initially characterized as 2-hydroxycyclopropyl-α-D-galactopyranoside (14), has now been shown to contain a sesquiterpene and β 1->5 linked galactofuranose units (4). Macko et al. (9) proposed a structure for HS toxin which contains two galactose units on each side of an aglycone residue ($C_{15}H_{24}O_2$), which can exist as three isomers. The three forms of the selective toxin were reported to cause runner lesions on susceptible sugarcane leaves.

The non-toxic compounds related to HS toxin can be produced by removal of galactose units from toxin. There are several isomeric forms of HS toxin and therefore of each of the lower molecular weight compounds. The lower mol wt compounds were called toxoids because they reduce the effects of HS toxin on susceptible tissue (5), and because they are structurally related to the toxin. Use of the term "toxoid" for these analogs is explained and justified in an earlier paper (7). An enzyme with β-galactofuranosidase activity has been isolated from cultures of H. sacchari (6,7). This enzyme may be, in part, responsible for removal of galactose from HS toxin and for accumulation of toxoids in culture fluids of the fungus. Toxoids I, II and III represent groups of isomeric forms that contain 1, 2 and 3 units of galactose, respectively (7). The primary objective of this study was to determine the

comparative effects of pretreatment with the three toxoids on toxin-induced losses of electrolytes. The toxoid most similar to HS toxin in structure gave maximum protection. To date, there are no data on production of toxoids in infected tissue.

MATERIALS AND METHODS

Isolates of H. sacchari were obtained from Jack L. Dean of the USDA Sugarcane Research Station at Canal Point, FL, Jack C. Comstock of the Hawaii Sugar Planters' Association, and Owen Sturgess of the Bureau of Sugar Experiment Station, Indooroopilly, Queensland, Australia. Stock cultures of the fungi were maintained on cane leaf agar (16). Cultures for toxin and toxoid production were grown in a modified Fries solution (10). Sugarcane clones NG 77-82, NG 77-234, NG 77-103, CP 73-1000 and Co 453 were obtained from J.L. Dean. Clones H52-4610 and H50-7209 were obtained from G.A. Strobel of Montana State University. Plants were grown in 5 gallon plastic pots in the greenhouse at temperatures between 18 and 24°C. The youngest fully-expanded leaves from plants of uniform size (1.5-2.5 meters high) were used in bioassays.

Purified toxin and toxoids were prepared by the use of activated charcoal plus thin layer, gel, and ion exchange chromatography, as previously described (4,7). Relative purity and cross contamination were monitored by GLC (7), which detects unwanted toxins and toxoids down to 1% (by wt) of the preparation. However, the procedure did not separate isomeric forms of each toxoid; thus, each preparation was a mixture of compounds with identical molecular weights. Concentrations of purified HS toxin and toxoids in solution were determined by dry weight (after

thorough drying at 110°C) and by measurement of the galactose released by acid hydrolysis of the toxin or toxoid, as previously described (7).

The assay to measure protective effects of toxoids was based on toxin-induced loss of electrolytes; this assay is more reliable than the assay based on development of runner lesions (12). Leaf disks (1.0 cm in diameter) were cut, immediately placed in water, and held for 3 h. Eight disks were then placed in each assay vial. The standard toxoid protection bioassay involved exposure of the disks to water (2 ml) or to a toxoid solution (2 ml) for one h; an aliquot (100 µl) of HS toxin solution was then added to bring the solution to the necessary toxin concentration. After exposure to toxin for 0.5 h, the disks were rinsed several times with water and placed in 5 ml of water (leaching solution). The disks were incubated on a shaker and the conductance of the solution was determined at intervals with a conductivity meter. Toxin or toxoid-induced loss of electrolytes was based on the conductance values of leaching solutions taken after 3 h incubation, less the values for the water controls. The level of leakage varied from assay to assay because of environmental factors prior to leaf harvest (1). Therefore, all assays included a series of known toxin concentrations for standards. Any variation in this procedure is specified in the results. Assays, which were run at 22° under laboratory lighting, were replicated at least three times; conductance values for replicates varied less than ±5% of the reported averages, unless stated otherwise. Abilities of toxin and toxoids to produce runner lesions on leaves was determined as described elsewhere (12,13). All experiments were repeated three or more times over a two year period.

RESULTS

Effect of excision and preincubation on sensitivity of leaf tissues to HS toxin. Variations between assays in rates of toxin-induced leakage of electrolytes were observed; accordingly, an attempt was made to determine the factors involved in variability. The rate of leakage was low when leaf disks were cut and immediately exposed to toxin. Rate of leakage was much higher when leaf disks were allowed to stand in distilled water at 23°C for 6 to 10 h prior to exposure to toxin (Fig. 1). Incubation in water for more than 10 h, prior to toxin treatment, resulted in decreases in toxin-induced loss of electrolytes. When a low concentration of toxin was used, a slightly longer incubation time may have been required to reach maximum sensitivity (Fig. 1). Greater sensitivity to toxin, expressed as higher rates of electrolyte loss and total losses of electrolytes, also was observed when leaf disks were washed for 0.5 h and held on wet filter paper (rather than on water) at 23°C for 6 h.

Effect of toxoid pretreatment on sensitivity of tissues to HS toxin. Leaf disks were cut and incubated in water for 6 h, then were exposed to toxoid III (100 µg/ml) for 0, 1, 2, or 3 h prior to toxin treatment. Toxoid pretreatment consistently gave protection against toxin; protection was maximum when toxoid III was applied one h before exposure to toxin. However, toxoid III gave protection even when tissues were exposed simultaneously to toxin and toxoid. Prior exposure to toxoids for more than one h gave erratic results.

Experiments were designed to determine whether or not maximum protection requires the presence of excess toxoid during toxin exposure. In a representative experiment, toxin at 5.0 and 1.0 µg/ml caused losses that gave conductance readings of 144 and 43 µmhos above the water

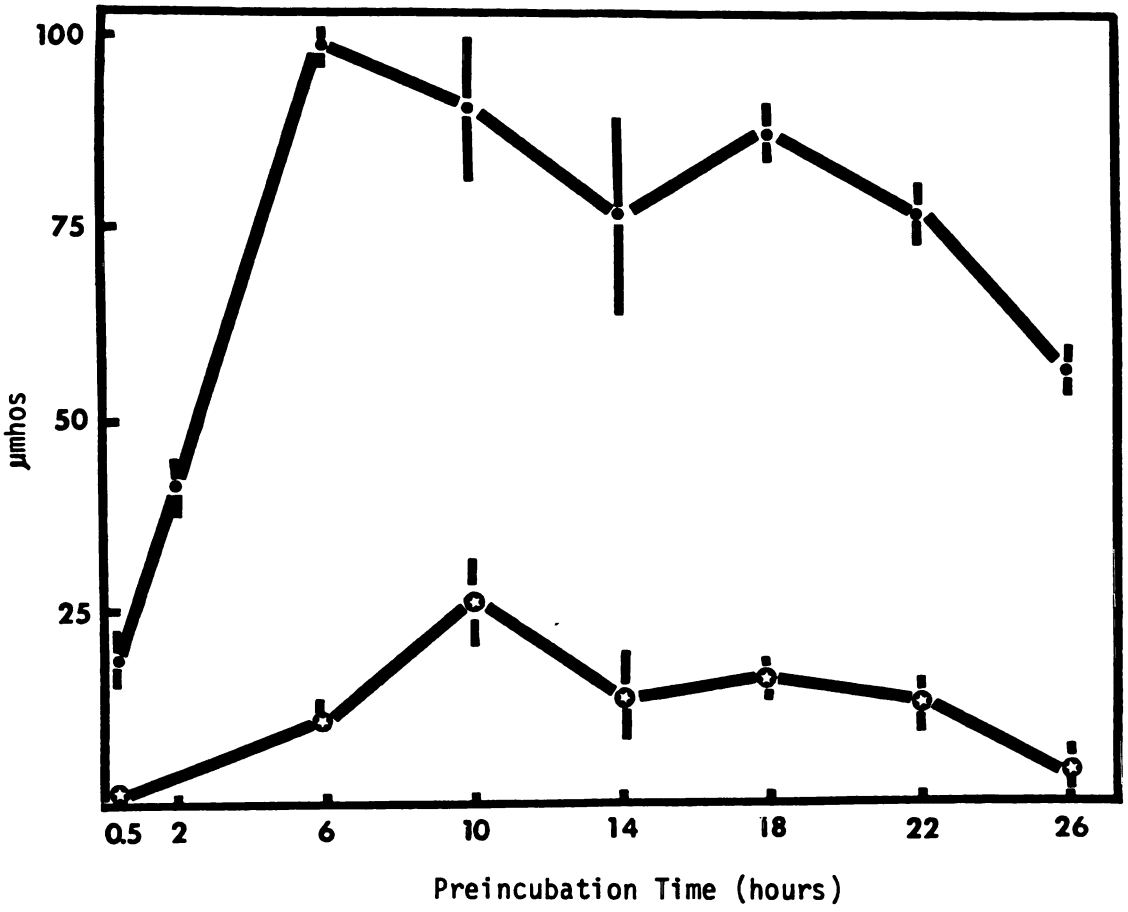

Fig. 1. Effect of preincubation in water on sensitivity of sugarcane (clone NG 77-82) leaf tissues to HS toxin, as determined by electrolyte leakage. Leaf disks were cut and held on water for the indicated times. Disks were then placed in toxin solution or in water for 0.5 h, rinsed, and placed in 5 ml leaching solution. Toxin-induced leakage was determined by conductance of the leaching solution at 3 h. Toxin was used at 0.15 µg/ml (✪) and 1.5 µg/ml (●). Standard deviation is shown by the vertical bars.

controls, respectively. Pretreatment of tissues with toxoid III (100 µg/ml) for one h, followed by exposure to the toxin (5 or 1 µg/ml) plus the toxoid for 1 h, gave conductance values in the leaching solutions of 5 and 0 µmhos, respectively. Thus, the toxoid gave essentially complete protection against toxin at both concentrations. This same procedure was repeated, except that the tissues were rinsed thoroughly after pretreatment with toxoid III, to remove free toxoid; no toxoid was added along with toxin. Electrolyte leakage was reduced well below that induced by toxin alone (no pretreatment with toxoid), although the protection was less than that obtained when toxoid was present in the toxin solution (Table 1). Therefore, much of the protective effect of a toxoid pretreatment appears to be retained even when excess toxoid solution is removed prior to toxin exposure.

The dosage-response curve for HS toxin-induced loss of electrolytes from clone NG 77-103 showed nearly linear increases in electrolyte losses with increases in toxin concentrations from 0.156 to 1.25 µg/ml (Fig. 2). None of the three toxoids caused loss of electrolytes from this clone of sugarcane. When leaf discs were treated with a toxoid prior to and during exposure to toxin, the amount of leakage was less than that induced by toxin alone. Toxoid III provided more protection than did toxoid II; toxoid I was the least effective (Fig. 3). Figures 2 and 3 show data from the same experiment and therefore can be compared. The standard curve of toxin-induced losses of electrolytes (Fig. 2) was used to calculate the apparent reduction in toxicity imposed by toxoids. For example, toxoid III at 100 µg/ml reduced the leakage induced by toxin at 1.25 µg/ml to the level that would be induced by toxin (without toxoid pre-treatment) at 0.08 µg/ml. This was calculated to be a 94% reduction

Table 1. Effect of pretreatment with toxoid III on HS toxin-induced losses of electrolytes from sugarcane leaves (clone Co 453). Leaf discs were cut, preincubated in H_2O, and held in toxoid solutions (2 ml, 100 µg toxoid/ml) (B, C) or water (A). Tissues were then rinsed (C) or not rinsed (B), toxin solutions were added, incubated for 1 h, rinsed, transferred to 5 ml water, and leached for 3 h.

Treatment	Conductance	
	5 µg toxin/ml	1 µg toxin/ml
	(µmhos)	(µmhos)
A. Water -> Toxin	144	43
B. Toxoid -> No Rinse -> Toxin	5	0
C. Toxoid -> Rinse -> Toxin	53	4

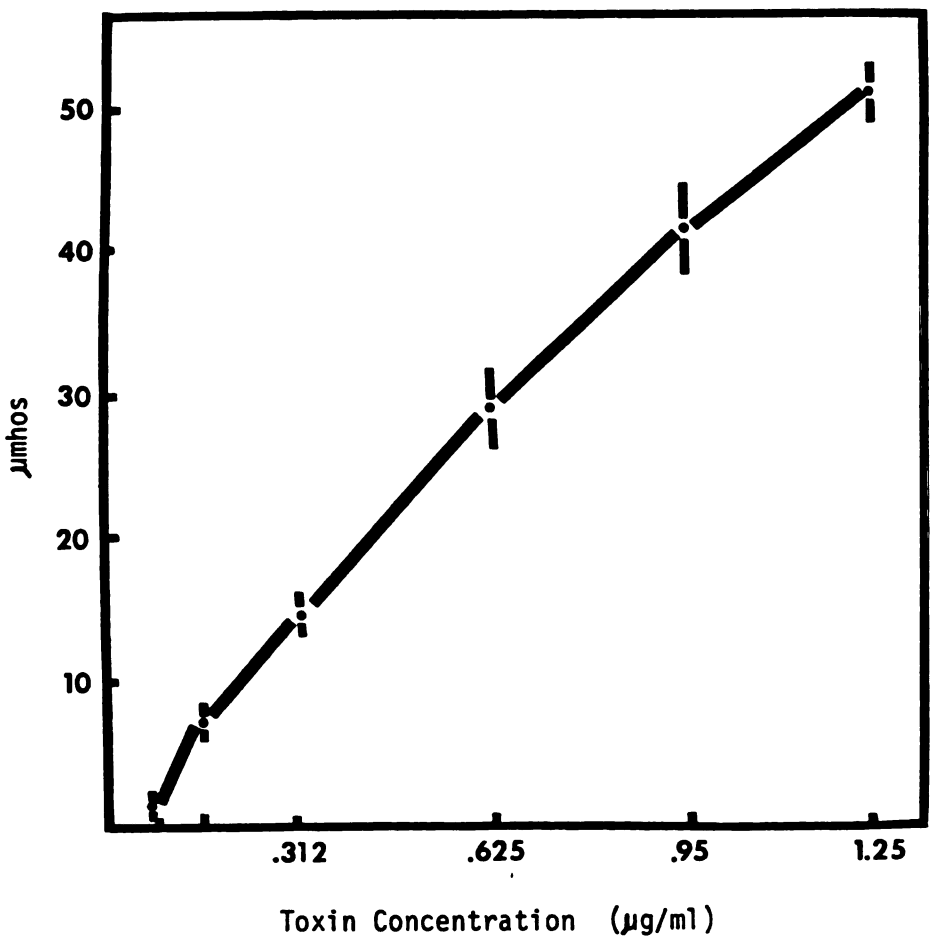

Fig. 2. Effect of HS toxin concentration on loss of electrolytes from leaf tissues of sugarcane clone NG 77-103. Leaf disks were cut, incubated on water for 4 h, exposed to toxin for 0.5 h, rinsed, and leached in 5 ml of water. The values are conductances of leaching solutions after 3 h, minus the water control. Standard deviation is shown by the vertical bars.

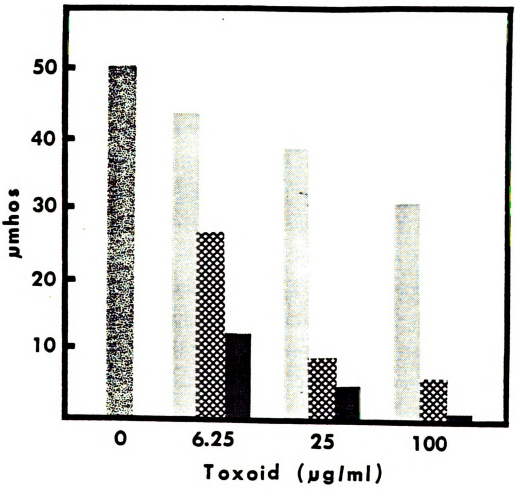

Fig. 3. Effect of toxoid pretreatment on HS toxin-induced leakage of electrolytes from leaf tissues of sugarcane clone NG 77-103. Leaf disks were cut, preincubated for 3 h, and exposed to toxoids I ( ), II ( ) or III ( ) for 1 h. Toxin was then added for a final concentration of 1.25 µg/ml; disks were then incubated for 30 min, rinsed, and leached for 3 h in 5 ml of water. Toxin-induced leakage is indicated by the conductance values. Each treatment was done in triplicate and the standard deviation is similar to that observed in Fig. 2.

in toxicity (Table 2). Toxoid III at 6.25 µg/ml gave 78% protection against toxin at 1.25 µg/ml. These percentages of protection provided by toxoids were calculated by the formula indicated in table 2.

The experiment was repeated with sugarcane clone Co 453. The leaves used in this experiment were in an unusually sensitive condition; as a result, HS toxin at 0.625 µg/ml caused maximum losses of electrolytes (Fig. 4). Toxin in this experiment was used at 1.25 µg/ml. Again, toxoid III provided more protection than did toxoid II, and toxoid I gave no detectible protection against toxin (Fig. 5). However, toxoid I gave measurable protection against toxin in other experiments with Co 453, using non-saturating levels of toxin (Table 3).

The data clearly show that prior exposure to toxoids can reduce HS toxin-induced loss of electrolytes. The degree of protection was directly proportional to the concentration of the toxoid used. Protection was greatest with the toxoid that was most similar to toxin.

Clonal differences in protective and toxic effects of toxoids. The three toxoid groups were tested for toxic and for protective effects against HS toxin, using five sensitive clones of sugarcane (Co 453, NG 77-103, NG 77-82, NG 77-234, and CP 73-1000). Tissues were pre-treated with toxoids in the usual way and then were exposed to non-saturating concentrations of toxin. Effects on electrolyte losses were determined 3 h after tissues were exposed to toxin and rinsed. Several control treatments were included: tissues exposed to water alone; tissues exposed to toxin without toxoids; and tissues exposed to each toxoid without toxin. The three toxoids, used alone at 100 µg/ml, caused no losses of electrolytes from tissues of clones Co 453 and NG 77-103. However, toxoid III used alone at 25 or 100 µg/ml caused leakage of

Table 2. Effect of pretreatment with toxoids on HS toxin-induced losses of electrolytes from sugarcane tissue (clone NG 77-103). Toxin was used at 1.25 µg/ml.

	Protective effects of toxoids					
	III		II		I	
Toxoid concentration	Appar. toxin[a]	Protection[b]	Appar. toxin	Protection	Appar. toxin	Protection
(µg/ml)	µg/ml	%	µg/ml	%	µg/ml	%
100	0.08	94	0.14	89	0.68	45
25	0.12	90	0.19	85	0.87	30
12.5	0.23	82	ND[c]	ND	ND	ND
6.25	0.27	78	0.58	53	1.03	18
0	1.25		1.25		1.25	

[a]Apparent toxin = Toxin-induced leakage equivalent to that for toxin at indicated levels. See Figs. 2 and 3.

[b] $\% \text{ Protection} = \frac{[\text{Actual Toxin}] - [\text{Appar. Toxin}]}{[\text{Actual Toxin}]} \times 100$

[c]Not determined.

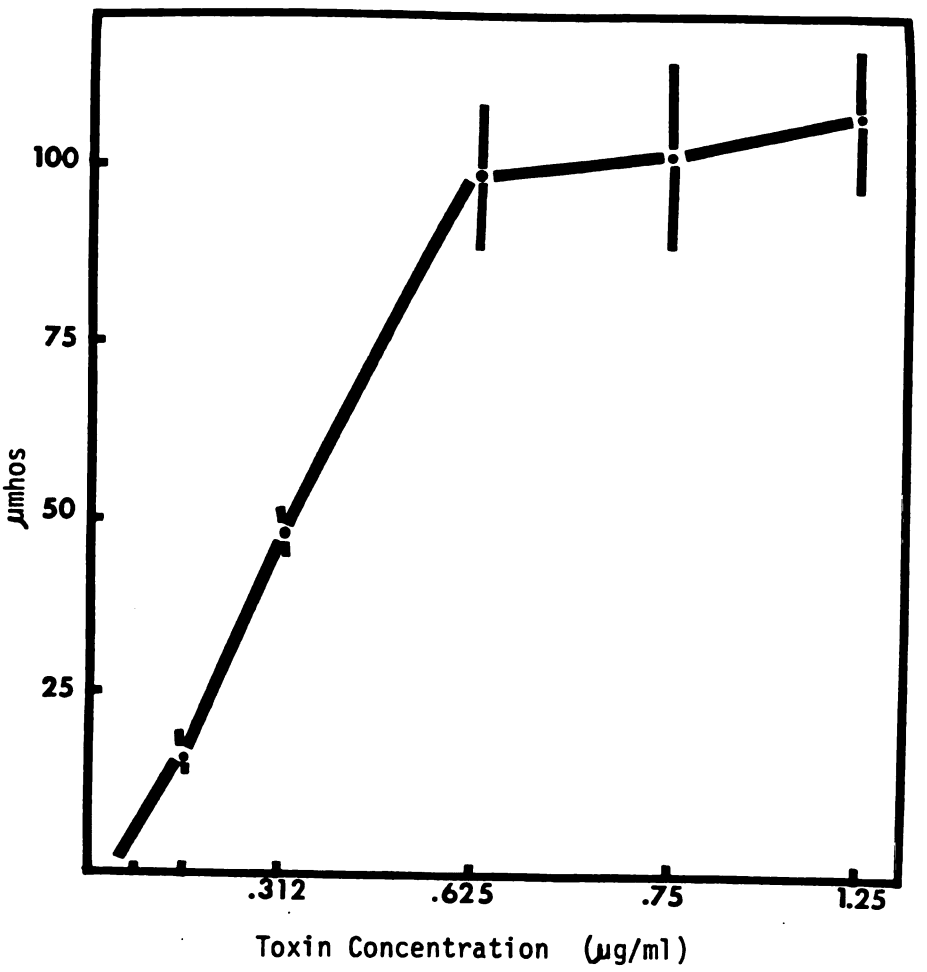

Fig. 4. Dosage-response relationship of HS toxin on leaf disks of sugarcane clone Co 453. Electrolyte leakage is indicated by conductance values of leaching solutions. Procedures are described in Fig. 2. Standard deviations are shown. Tissues used in this experiment were in an unusually sensitive condition, because of environmental conditions during growth.

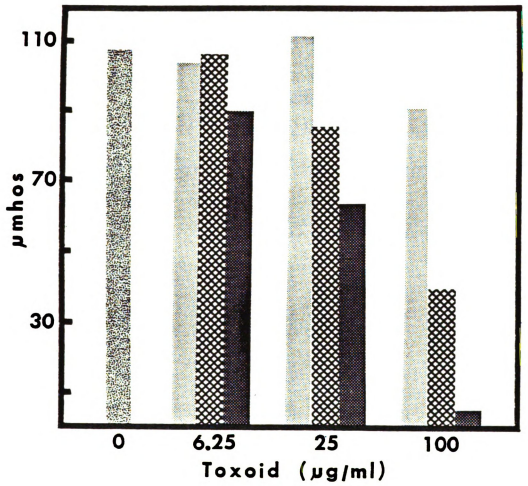

Fig. 5. Effect of toxoid pretreatment on HS toxin-induced leakage of electrolytes from leaf tissues of sugarcane clone Co 453. Toxoids I (▒), II (▓), and III (■) were used. Toxin concentration was 1.25 µg/ml. Other procedures are described in Fig. 3.

Table 3. Protective effects of toxoids against activity of HS toxin, plus toxic effects of toxoid III against certain clones. Protection was measured by effects of pretreatment on toxin-induced loss of electrolytes.

		Conductance (μmhos)						
		Toxoid pretreatment and concentrations (μg/ml)						
		None[a]	III		II		I	
Sugarcane clone	Toxin concentration	0	25	100	25	100	25	100
	μg/ml							
NG 77-103	1.25	51	4.5	1	8.5	6	39	32
	0		0	0	0	0	0	0
Co 453	0.625	62	11	1	21	8	55	40
	0		0	0	0	0	0	0
NG 77-82	0.625	68	65	50	24	8	75	44
	0		45[b]	51	0	0	0	0
NG 77-234	1.25	99	103	108	50	32	100	76
	0		ND[c]	106	ND	6	ND	0
CP 73-1000	2.5	65	58	52	57	29	72	64
	0		20	25	0	2	0	2
H52-4610[d]	100.	0	ND	0	ND	0	ND	0
	0	0	ND	0	ND	0	ND	0
H50-7209[d]	100.	0	ND	0	ND	0	ND	0
	0	0	ND	0	ND	0	ND	0

[a]Toxin-induced electrolyte loss with no pretreatment.

[b]Toxoid-induced electrolyte loss with no exposure to toxin.

[c]ND = not determined.

[d]Clones H52-4610 and H50-7209 are resistant to H. sacchari; all others are susceptible.

electrolytes from tissues of clones NG 77-82, NG 77-234, and CP 73-1000 (Table 3). Toxoid III-induced losses were smaller than the HS toxin-induced losses; for example, the leaching solution for clone NG 77-82 exposed to toxoid III at 100 µg/ml had a conductance value of 51 µmhos whereas the value for toxin at 0.625 µg/ml was 68 µmhos. The conductance value for toxin-treated tissue (65 µmhos) was less than half the value induced by a concentration of toxin which induced the maximum rate of electrolyte loss.

Leaching solutions for tissues that were pretreated with toxoid III (100 µg/ml) and exposed to HS toxin (0.625 µg/ml) had a conductance value of 50 µmhos (Table 3). Therefore, the tissues of clone NG 77-82 that were exposed to toxin did not leak more than that induced by the toxoid III alone. This showed that toxoid III induced some losses of electrolytes from tissues of clone NG 77-82, but the toxoid also protected against further toxin-induced losses. A lower level of toxoid III (25 µg/ml) induced even less electrolyte loss and provided less protection against toxin-induced loss of electrolytes. In contrast to toxoid III, toxoids II and I induced little or no loss of electrolytes from the sugarcane clones tested. Resistant clones (H52-4610 and H50-7209) were insensitive to HS toxin (100 µg/ml) and to the toxoids (100 µg/ml).

Tests showed that both HS toxin and toxoid III (100 ng each) caused runner lesions on leaves of sugarcane clones NG 77-82 and NG 77-234. Leaves of clones Co 453 and NG 77-103 developed runner lesions in response to toxin but not to toxoid III. Experiments are underway to isolate the 6 isomeric forms of toxoid III and to determine which forms give maximum protection and which will induce losses of electrolytes from selected clones of sugarcane.

DISCUSSION

Greenhouse-grown sugarcane can vary in sensitivity to HS toxin, as indicated by toxin-induced loss of electrolytes. Greenhouse temperature is a major factor in this variability (1); the temperature of the greenhouse must be maintained below 24°C for tissues to have maximum sensitivity to toxin. Another source of variability was traced to the treatment of leaf disks used in bioassays. Leaf disks that were cut and exposed immediately to toxin leaked much less than did disks that were held for 6 h in water prior to exposure to toxin. Also, the minimum concentration of toxin that induced leakage in the preincubated disks was much less than the minimum required by the freshly-cut disks. All these sources of variability must be controlled for accurate data in toxin bioassays and in toxoid protection experiments.

The ability of toxoids to protect leaf tissues against subsequent losses of electrolytes induced by HS toxin was examined. Protective effects of toxoids were evident when tissues were pre-treated with toxoids or treated concomitantly with toxoids plus toxins. However, pre-treatment may leave significant amounts of toxoids in intercellular spaces. Therefore, an attempt was made to remove as much toxoid as possible, by thorough washing prior to toxin exposure. Toxoids gave somewhat more protection by pre-treatment without washing, but most of the protective effect was evident in the thoroughly washed tissues. These results suggest binding between toxoid and a putative receptor; such binding could reduce the subsequent interaction of toxin with a receptor. Binding of toxin to a protein in susceptible sugarcane was claimed in earlier work (15), but attempts to repeat the crucial experiments were not successful (2). The toxoid data indicate that

binding sites should still be considered. However, mechanisms other than the simple competition of HS toxin and toxoid for a common receptor site could be involved.

Toxoid III, which has 3 units of galactose and is most similar to HS toxin, gave better protection against toxin than did the other toxoids, as determined by toxin-induced losses of electrolytes. Toxoid II (2 units of galactose) gave protection, but was less effective than was toxoid III. Toxoid I (1 unit of galactose) which is least like toxin in structure, gave little protection against toxin. The correlation of structural similarity to toxin and ability to protect tissues against toxin-induced losses of electrolytes supports the hypothesis that the toxoids are competitive inhibitors of HS toxin. The toxin and toxoid preparations used in these studies can be separated by HPLC into several isomeric forms (9). Preliminary data indicate that the isomers may not behave identically (8). A more detailed kinetic analysis involving the HPLC-purified isomeric forms of toxin and toxoids will be necessary to determine whether or not the toxoids are competitive inhibitors of toxin.

The molecular weight of HS toxin and toxoids were not known when these experiments were completed, but the values are now available. On a molar wt basis, the ratio of toxoid to toxin required for 90% protection was 24:1 for toxoid III and 140:1 for toxoid II. A ratio of 180:1 for toxoid I gave only 45% protection. These values are calculated from the data given in Fig. 3.

Earlier studies (5) indicated that the toxoids did not induce loss of electrolytes from certain clones of sugarcane (CP 52-68, NG 77-103, Co 453) that are sensitive to HS toxin. When the toxoids were tested for

ability to induce loss of electrolytes from other toxin-sensitive clones, three (NG 77-234, NG 77-82, CP 73-1000) were found that were sensitive to toxoid III. This toxoid also caused runner lesions to develop on these same toxin-sensitive clones, but not on the other toxin-sensitive clones, and not on H. sacchari-resistant clones. However, the natural mixture of toxoid III isomers caused less damage on a weight basis (as measured by electrolyte loss and production of runner lesions) than did HS toxin. Although toxoid III induced electrolyte losses, it also protected tissues against further toxin-induced losses of electrolytes. The various toxin analogs may be used to determine the structural requirements for maximum affinity to sensitive sites, and for toxic action. These findings could have relevance to an understanding of disease development in certain clones.

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EXPERIMENTAL IV

SELECTIVE TOXINS AND ANALOGS PRODUCED BY *HELMINTHOSPORIUM SACCHARI*: PRODUCTION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

SELECTIVE TOXINS AND ANALOGS PRODUCED BY HELMINTHOSPORIUM SACCHARI: PRODUCTION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

Abstract

Helminthosporium sacchari (HS) toxin and several lower mol wt, non-toxic analogs (toxoids) were isolated from culture filtrates. Three isomers of the toxin (A, B, and C) were separated by HPLC. Each differed from the others in relative toxicity to susceptible sugarcane; resistant sugarcane was not affected. Next, each toxin isomer was partially digested with a β-galactofuranosidase and the resulting toxoids (seven from each toxin isomer) were separated by reverse phase HPLC and identified. Each isomer of toxoid III (three galactose units/mol) also was partially digested and the arrangement of gal units was determined. One of the toxoids ($A_{1,2}$, which has one gal on left and 2 on right of sesquiterpene A) was found to be toxic to certain clones of H. sacchari-susceptible sugarcane, but not to other susceptible clones. This toxoid was derived from the least active form of toxin (form A); nevertheless, toxoid $A_{1,2}$ was as toxic to certain clones as was the most active form of toxin (form C). The degree of protection obtained with each of the six toxoid III isomers was determined by use of the electrolyte leakage assay. Toxoid $C_{2,1}$ was more effective at preventing toxin C-induced electrolyte losses than was any other toxoid. Each isomer of toxoid III protected better than did any isomer of toxoid II (two gal units/mol). Toxoids with the 1,1 gal arrangement did not protect as well as did

toxoids with the 2,0 or 0,2 gal arrangement. Thus, the sesquiterpene isomer, the number of gal units, and the gal arrangement pattern determine the effectiveness of the compound in induction of electrolyte loss and in prevention of toxin C-induced electrolyte loss from sugarcane tissues.

INTRODUCTION

Four plant pathogenic fungi from the genus Helminthosporium are known to produce selective toxins (16) which are active only against host genotypes. The genotypes that are sensitive to toxin are always susceptible to the pathogen which produced that toxin. Several of these toxins have been isolated and characterized (13). It is becoming apparent that the toxins, as produced in culture, exist in several different but closely related forms (1,2,4,7). There are also toxin analogs which can be either non-toxic or selectively toxic; certain analogs have a more narrow selectivity than do the major toxins (8).

H. sacchari produces toxin (4 gal/mol) in three isomeric forms. Each form contains a different isomer of a sesquiterpene ($C_{15}H_{24}O_2$) (11). Attached to this unsymmetrical sesquiterpene are 2 groups, each composed of two β 1,5-linked galactofuranose units. Non-toxic, lower mol wt analogs, termed toxoids (7), have been found in the culture fluids of H. sacchari. The toxoids can be grouped by the number of galactose units in the molecule. Toxoids III, II and I were shown to contain 3, 2 and 1 units of galactose, respectively (7).

A β-galactofuranosidase produced by H. sacchari was shown to convert HS toxin to toxoids in vitro. The studies also suggested that this enzyme probably converts toxin to toxoids in cultures (7). Sequential

removal of one, two, or three galactose units from the three sesquiterpene isomers of toxin should give 21 different, lower mol wt toxoids. We have examined this situation by enzymatic removal of galactose units from each form of toxin, and have identified the resulting compounds by chromatography.

A previously reported method of nomenclature for the toxoids was followed (10). The toxin identification letter (A, B, or C) refers to the sesquiterpene core isomer present in the molecule. The subscript numbers refer to the number of galactose units present and their position in relation to the core. The first number refers to galactose attached to the left side of the molecule and the second number to galactose on the right side of the sesquiterpene. For example, toxoids $C_{2,1}$ and $C_{1,2}$ were produced by removal of a single galactose unit from toxin $C_{2,2}$.

Previous work showed that toxoids with 3 galactose units protect tissues from toxin more effectively than do the 2 galactose toxoids, as shown by the electrolyte leakage assay. A small but detectable level of protection was provided by the toxoids with 1 galactose unit (9). However, in each case the three, two, and one galactose toxoids were a mixture of isomers. These mixtures of toxoids have now been separated by reverse phase HPLC. The protective and toxic ability of several of these purified toxoids will be reported here. Some of this work was reported in an abstract (8).

MATERIALS AND METHODS

Isolates of Helminthosporium sacchari and the sugarcane clones used were obtained from previously reported sources (7). Toxins and toxoids were isolated from cultures of H. sacchari by a modified version of the

procedure described previously (5). The initial separation between toxin and the toxoids was achieved on a Sephadex LH-20 column as previously described. The purification procedure was then modified by using flash chromatography instead of TLC and by including reverse phase HPLC as the final step. The toxin and toxoid content of each fraction from the LH-20 column was identified and measured by GLC (7). For flash chromatography, 30 gm of Whatman LPS-2 was poured as a slurry in acetonitrile into a column giving a bed with dimensions of 2.2 X 19 cm. A methanol solution of the preparation to be chromatographed was mixed with 1.5 gm of LPS-2 and gently stirred while the solvent evaporated. The final powder was applied to the top of the column bed and the sample was eluted, under pressure, with stepwise gradient solutions consisting of 100 ml volumes of acetonitrile-water in the following ratios: 100:0, 97.5:2.5, 95:5, 90:10, 85:15 and finally 80:20. Fractions (9 ml) were collected and monitored for toxin and toxoid content by TLC as previously described (5).

A Varian 5000 Liquid chromatography instrument equipped with a Waters μBondapac C_{18} column (0.78 X 30 cm) was used for HPLC. Toxins and toxoids isolated from the culture fluids were chromatographed with acetonitrile-water mixes, as follows: for toxins, 20:80, for toxoids III, 19:81, for toxoids II', 20:80, and for toxoids II, 26:74. The flow rate was 2 ml/min. The change in absorbance of the eluate at 215 nm was monitored and fractions were collected manually. A second method was used for obtaining purified toxoids for determination of biological activities. HPLC-purified toxins A, B, and C (60 mg of toxin C, 20 mg each of toxins A and B) were partially hydrolyzed at 37° for 1.5 hr in a 1 ml solution of β-galactofuranosidase from Penicillium charlesii. The

resulting solution of toxoids was evaporated to dryness, the residue was dissolved in 26% acetonitrile, and the solution chromatographed by HPLC as described above. The eluate containing each toxoid isomer was collected, concentrated, and dissolved in methanol for storage.

To determine the concentrations of purified toxin and toxoids in solutions, the amounts of galactose released by acid hydrolysis were measured by a modified version of the procedure by Fischer and Zapf, as previously described (7). The calculations were based on molecular weights of toxin and toxoids as follows: 884 for toxin (4 gal/mol); 722 for toxoids III (3 gal/mol); 560 for toxoids II (2 gal/mol); 398 for toxoids I (1 gal/mol).

Purified toxins A, B and C and each isomer of toxoid III were partially digested using β-galactofuranosidase isolated from Helminthosporium sacchari, H. maydis (7), or Penicillium charlesii (14). This enzyme was partially purified from the culture fluids of P. charlesii and H. maydis, and from the mycelium of H. sacchari (7). The procedure involved concentration of the crude enzyme solution (160 ml) to a volume of 32 ml, precipitation of the proteins with ammonium sulfate at 75% of saturation, and extensive dialysis of the resuspended enzyme pellet against 2.5 mM acetate buffer (pH 4.6). The volume of the final preparation was adjusted to 20 ml. Toxins A, B, and C (300 μg of each) were dissolved in 270 μl of 2.5 mM acetate buffer (pH 4.6) and 30 μl of enzyme solution was added. The preparation was partially digested at 37° for 40 minutes for toxin C, and 30 minutes each for toxins A and B. The reaction was stopped by adding 0.5 ml of methanol and the preparation was evaporated at 45° under a jet of nitrogen. The residue was then dissolved in 300 μl of 20% acetonitrile in water. Aliquots (50 μl) of

the hydrolyzed solution of each isomer of toxin were then subjected to HPLC, using a program of 0-5 minutes acetonitrile-water (20:80), followed by a 10 min linear gradient to 27% acetonitrile and was then maintained at 27% for 20 min. Other conditions for HPLC were as stated above.

The six toxoid III isomers were also purified from culture fluids. A sample of each isomer (50 μg) was then dissolved in 90 μl of acetate buffer (2.5 mM, pH 4.6) and 10 μl of the Penicillium enzyme preparation was added. After 25 minutes at 37°, the sample was prepared for HPLC as was done for the partial digests of toxin. The residue was dissolved in 80 μl of 20% CH_3CN. Aliquots (40 μl) were chromatographed by HPLC using the same gradient used for analysis of the partial digests of the toxins.

The biological activities of the toxins and toxoids were measured with the electrolyte leakage assay (15). The assay is based on induction of leakage from susceptible leaf tissue by toxin, and prevention of toxin-induced losses by the toxoids. Leaf disks that were cut from young but fully expanded sugarcane leaves were preconditioned by incubation in water for 6 hr (9). In protection assays the toxin and toxoid solution was pre-mixed to provide simultaneous exposure of the disks. After 0.5 hr of exposure to the test solution, the disks were rinsed and placed in 5 ml of water (leaching solution). Toxin or toxoid-induced loss of electrolytes was based on the conductance values of leaching solutions taken after 3 hr incubation, minus the values for the water control. The ability of toxin and toxoids to produce a runner lesion was tested as previously described (15). Resistant tissue controls were used in all assays.

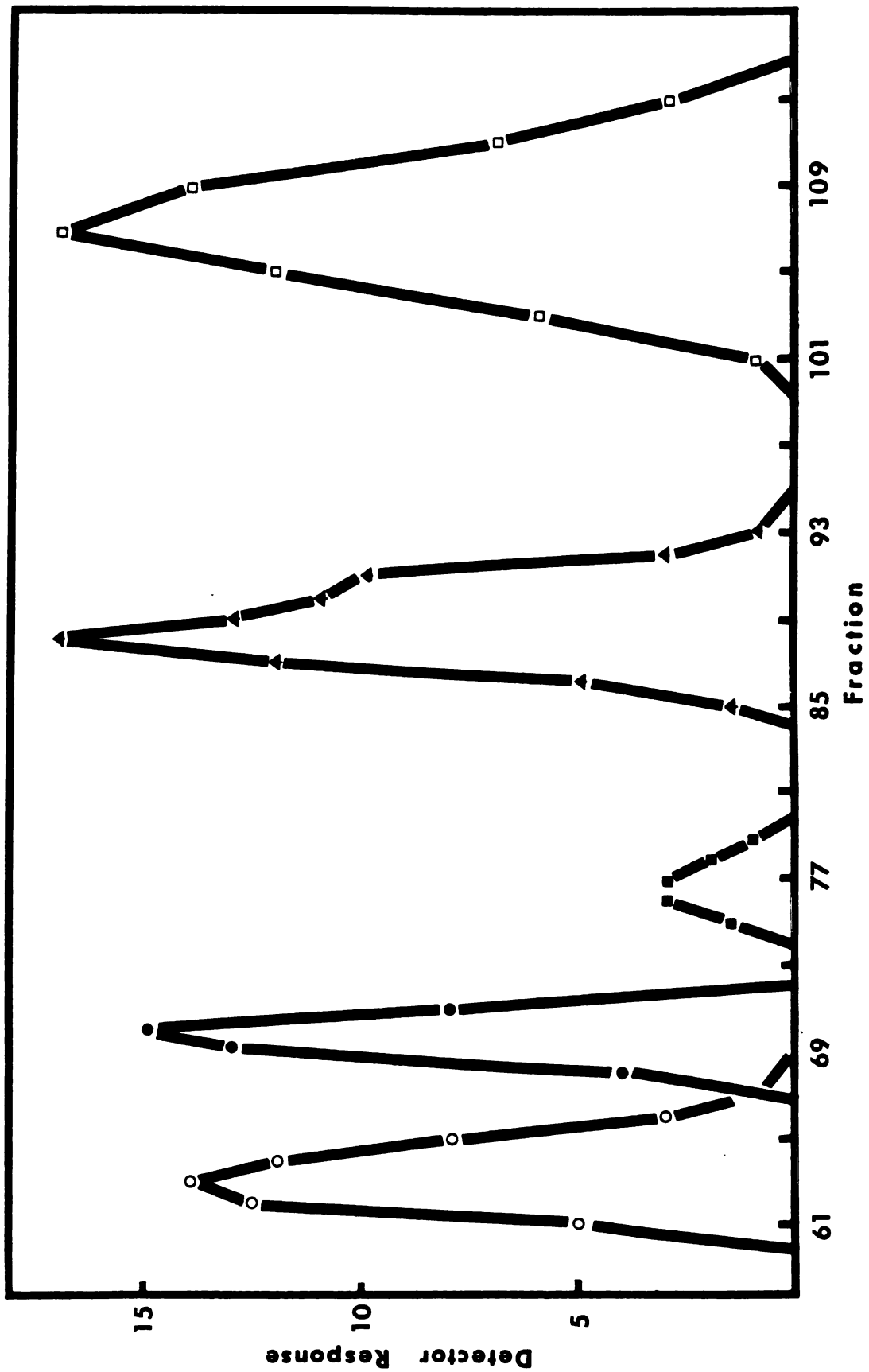
All solvents were redistilled, acetonitrile was HPLC grade, and water was double distilled. Each treatment was done in triplicate and each assay was repeated. All numerical values are averages from one experiment. The variation for each treatment never exceeded ±5% of the average.

RESULTS

Isolation and identification of toxins and toxoids. Toxoids obtained by removal of galactose (gal) are more hydrophobic than is the parent toxin. Sephadex LH-20, being lipophilic, gave a more dramatic separation of the toxin and toxoids than was achieved on a gel permeation column. Therefore, chromatography on an LH-20 column was used as an early step in the purification procedure. GLC of an aliquot of each fraction was used to measure the toxin and toxoid elution profile from the LH-20 column (Fig. 1). Aliquots of fractions from each peak were also subjected to HPLC. The results indicated that the LH-20 column separated these compounds primarily on the basis of number of gal units/molecule (data not shown). All isomers of each toxoid group eluted as a single peak. One exception was the peak labeled toxoid II'. This peak contained the 3 sesquiterpene isomers of toxoid II that had each gal unit attached directly to the sesquiterpene ($A_{1,1}$,$B_{1,1}$,$C_{1,1}$). Sesquiterpene and gal conformational isomers of each toxoid group were separated successfully by use of reverse phase HPLC.

HPLC was used also for separating the 3 isomeric forms of toxin (Fig. 2) which differ from each other in their absorbance at 215 nm. The activities of the three forms of toxin were compared using the electrolyte leakage bioassay. All three forms were toxic to susceptible

Fig. 1. Elution pattern of HS toxin (○) and toxoids III (●), II' (■), II (△), and I (□) from a Sephadex LH-20 column (80x3 cm). The eluent was 50% methanol and 7 ml fractions were collected. An aliquot of each fraction was derivatized and the toxin and toxoids present were determined by GLC (5). The height of each peak indicated amounts.


Detector Response
15
10
5
61
69
77
85
93
101
109
Fraction

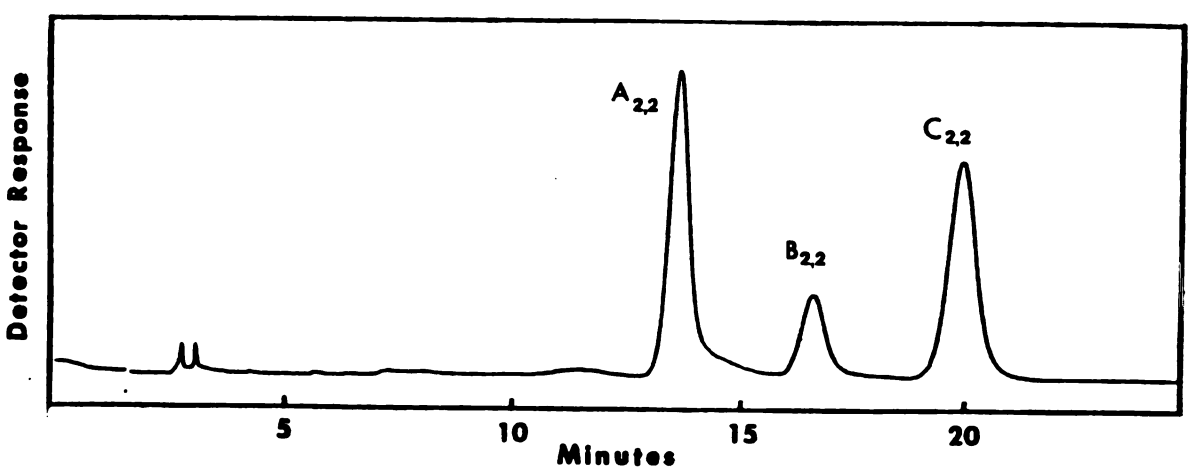


Fig. 2. Elution of the three forms (A, B, and C) of HS toxin from reverse phase HPLC following application of 10 µg each to the column. The eluent was 20% acetonitrile in water and the flow rate was 2 ml/min. Absorption at 215 nm was monitored.

sugarcane but had no measurable effect at 100 µg/ml on resistant sugarcane. Toxin C was more active than were the two other forms and toxin B was slightly more active than was toxin A (Fig. 3). Saturating concentrations of each toxin induced the same rate of electrolyte loss for the first 4 hr following exposure to toxin (data not shown).

The large number of toxoids found in the culture fluids of H. sacchari made separation and identification difficult. To simplify the procedure, toxins A, B, and C were separated and then partially digested with the β-galactofuranosidase from Penicillium charlesii. The chromatographic profile of each partial digest of each form of toxin (A, B, and C) should contain seven toxoid peaks (Figs. 4, 5), each with the same sesquiterpene isomer. However, the two toxoid I isomers with the A form of the sesquiterpene had nearly the same retention times and eluted as a single peak; as a result only six toxoid peaks were seen. Removal of either of the two terminal galactose units from each form of toxin produced two isomers of toxoid III (Fig. 5). The following question was then posed: how is the galactose arranged in each of the toxoids? This was answered in part by use of partial digests of each of the six toxoid III isomers and identification of the resulting toxoids (Table 1). These data do not tell whether the galactose is attached to the left or the right side of the sesquiterpene core; i.e., the data do not tell whether the galactose is arranged 2,1 or 1,2. The data only show that two galactose units are on one side and one galactose unit is on the other side of the sesquiterpene. Because of this, we have adopted the galactose arrangement for the toxoid III isomers as indicated by Macko (10, and personal communication).

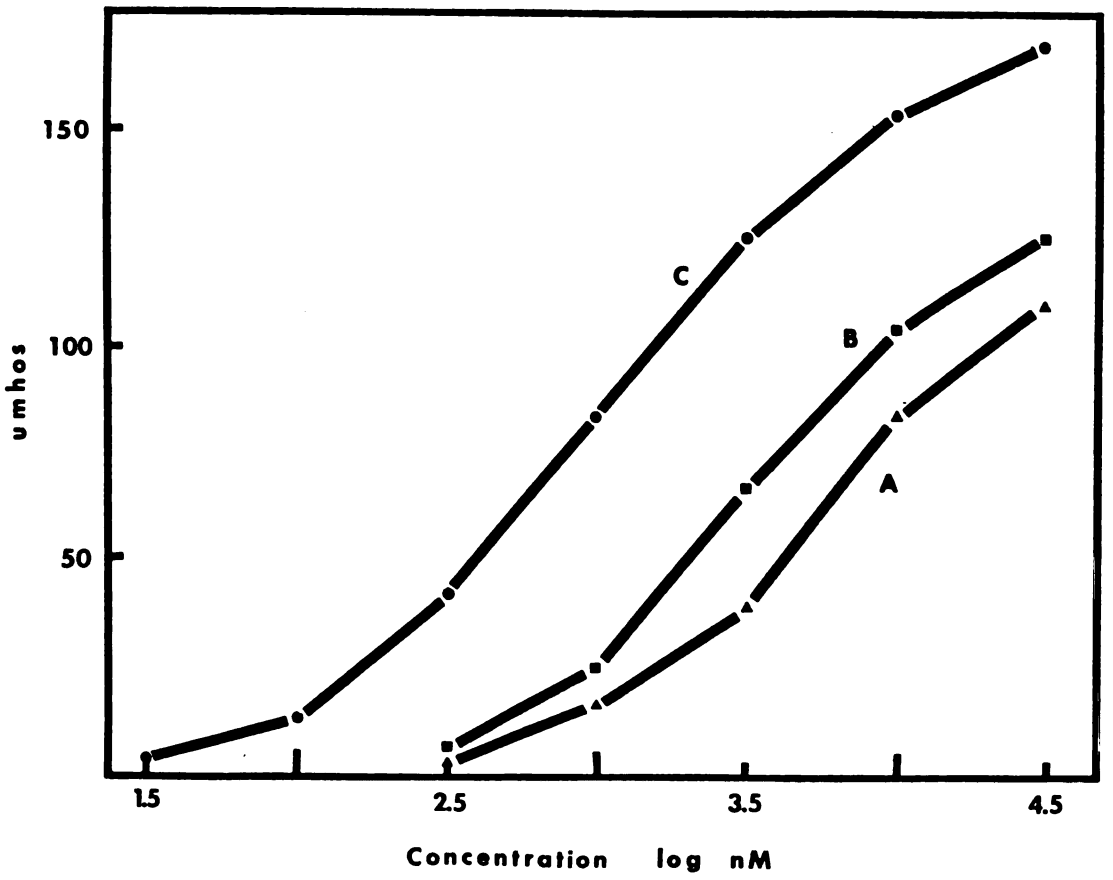
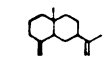


Fig. 3. Electrolyte leakage induced by each of the three forms of HS toxin. Leaf disks of sugarcane clone NG 77-234 were exposed to toxin for 0.5 hr, rinsed, and incubated in 5 ml of water. Conductance of the leaching solution was measured after 3 hr.

Fig. 4. Toxoids produced from toxin C by enzymatic removal of galactose units. The sesquiterpene in toxin A [] and B [] differs in arrangement of a double bond (11). Toxins A and B each produce a set of toxoids comparable in galactose arrangements to those shown above.

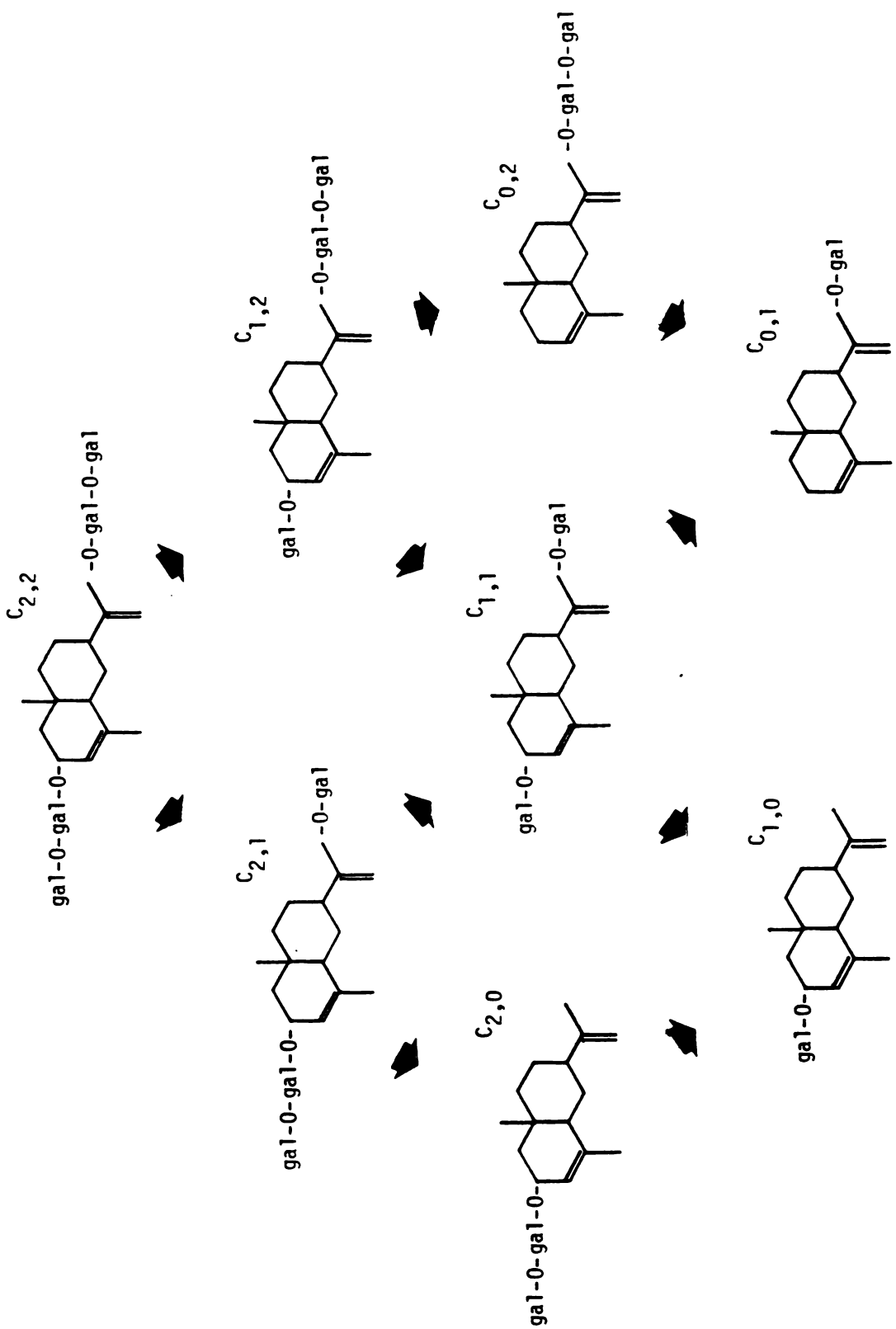

$C_{2,2}$
gal-O-gal-O-
-O-gal-O-gal
$C_{1,2}$
gal-O-
-O-gal-O-gal
$C_{0,2}$
-O-gal-O-gal
$C_{2,1}$
gal-O-gal-O-
-O-gal
$C_{1,1}$
gal-O-
-O-gal
$C_{0,1}$
-O-gal
$C_{2,0}$
gal-O-gal-O-
$C_{1,0}$
gal-O-

Fig. 5. HPLC separation of toxoids resulting from partial digestion of each of the 3 forms of HS toxin. The β-galactofuranosidase produced by P. charlesii was used for digestion. The letter in each panel indicates the sesquiterpene isomer present in the toxin and toxoids. Each peak is labeled to indicate the number and arrangement of galactose in each compound. HPLC conditions are described in material and methods.

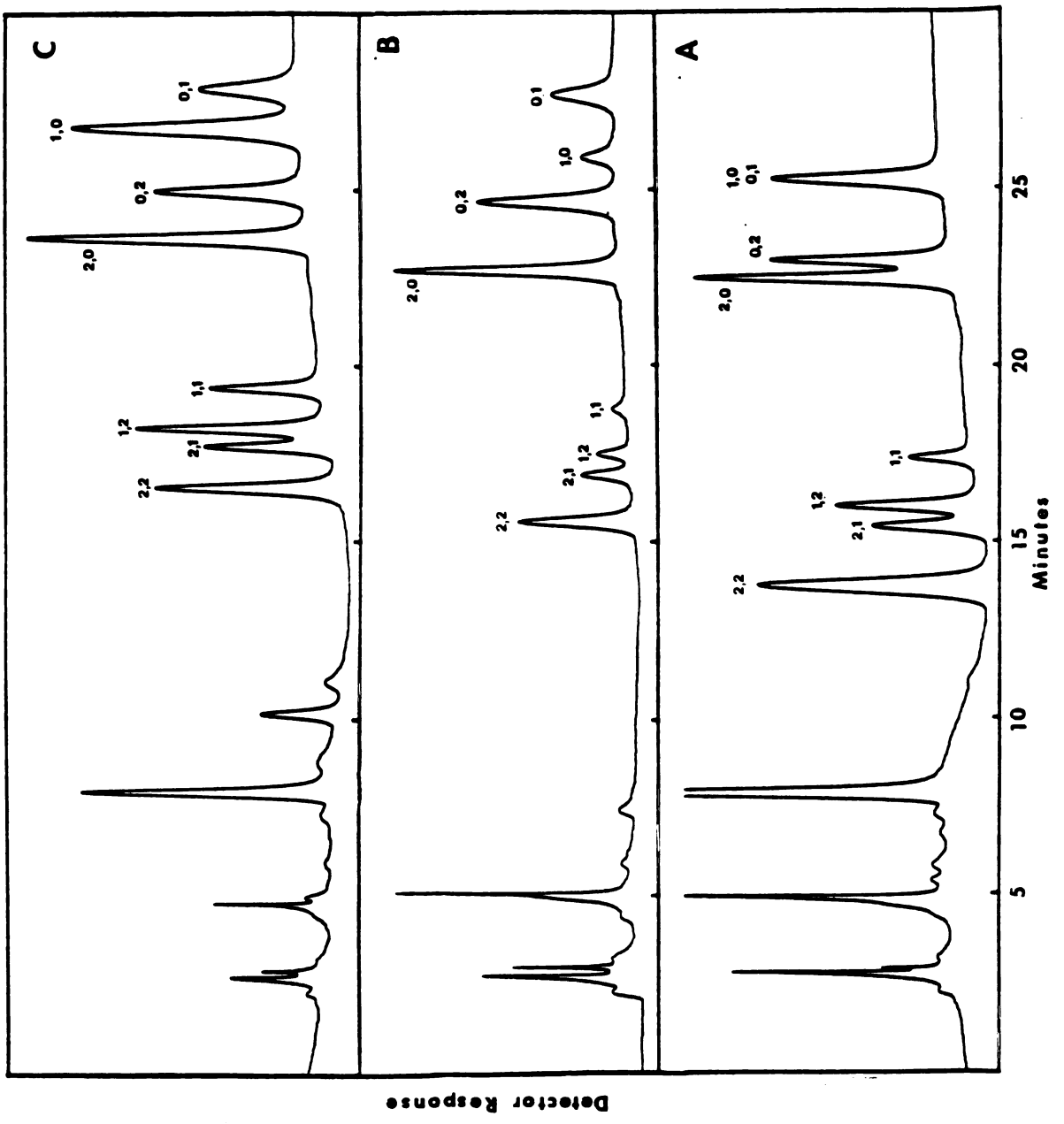

C
B
A
2,0
2,2
2,1
1,2
1,1
0,2
1,0
0,1
5
10
15
20
25
Minutes
Detector Response

Table 1. Relative amounts of toxoids obtained from partial digestion of each of the six isomers of toxoid III, as determined by peak heights. Toxoid III was digested and the resulting toxoids were separated by HPLC and identified by their retention times. The galactose arrangement for the six isomers of toxoid III were determined by Macko (10).

Substrate		Peak Heights (cm) and Toxoid Assignments[1]						
	Retention times (min)	15.3	16.	17.3	22.1	22.8	25.1	25.2
$A_{2,1}$		10	0	1	30	0	8.5	0
$A_{1,2}$		0	13	1	0	9.5	0	6.0
	Toxoid Assignments	$A_{2,1}$	$A_{1,2}$	$A_{1,1}$	$A_{2,0}$	$A_{0,2}$	$A_{1,0}$	$A_{0,1}$
	Retention times (min)	17.	17.5	19.1	22.9	25.	26.2	28.
$B_{2,1}$		1.5	0	0.25	13	0	2	0
$B_{1,2}$		0	2.5	1	0	11	0	6.5
	Toxoid Assignments	$B_{2,1}$	$B_{1,2}$	$B_{1,1}$	$B_{2,0}$	$B_{0,2}$	$B_{1,0}$	$B_{0,1}$
	Retention times (min)	18.2	18.7	19.8	24.4	26.1	27.9	29.4
$C_{2,1}$		7.5	0	4	20	0	10.5	1.0
$C_{1,2}$		0	15.5	7	0	12.	4.5	6.0
	Toxoid Assignments	$C_{2,1}$	$C_{1,2}$	$C_{1,1}$	$C_{2,0}$	$C_{0,2}$	$C_{1,0}$	$C_{0,1}$

[1]See text.

The arrangements of galactose units in toxoids I and II were ascertained from the arrangement in the toxoid III isomers. For example, removal of one of the terminal galactose units from toxoid $C_{2,1}$ will produce $C_{2,0}$ or $C_{1,1}$ but not $C_{0,2}$ (see Fig. 4). Therefore, the toxoid III isomer not present in the partial digest of toxoid $C_{2,1}$ must be toxoid $C_{0,2}$. The same logic was used to identify toxoid $C_{2,0}$ after partial digestion of toxoid $C_{1,2}$. The toxoid III isomer that was present in the partial digests of both $C_{2,1}$ and $C_{1,2}$ must be toxoid $C_{1,1}$. Next, the galactose arrangement of the two toxoid I isomers containing the C sesquiterpene was determined. The predominate isomer of toxoid I in the partial digest of $C_{2,1}$ must be $C_{1,0}$. This is because it is produced by further hydrolysis of both $C_{2,0}$ and some of the $C_{1,1}$. The small amount of $C_{0,1}$ is produced only from $C_{1,1}$ because no $C_{0,2}$ was found in the partial digest of toxoid $C_{2,1}$. Again, the same logic was used to identify toxoid $C_{0,1}$ in the partial digest of toxoid $C_{1,2}$.

The galactose arrangement for the toxoids resulting from digestion of the other forms of toxoid III, which contain the A and B sesquiterpene isomers, were determined by the same logic that was used for the C isomers. Each isomer of toxin (A, B, and C) was also partially digested with the β-galactofuranosidase isolated from H. maydis and H. sacchari. The same toxoids, although in different relative amounts, were produced using these enzymes as the toxoids produced with P. charlesii enzyme.

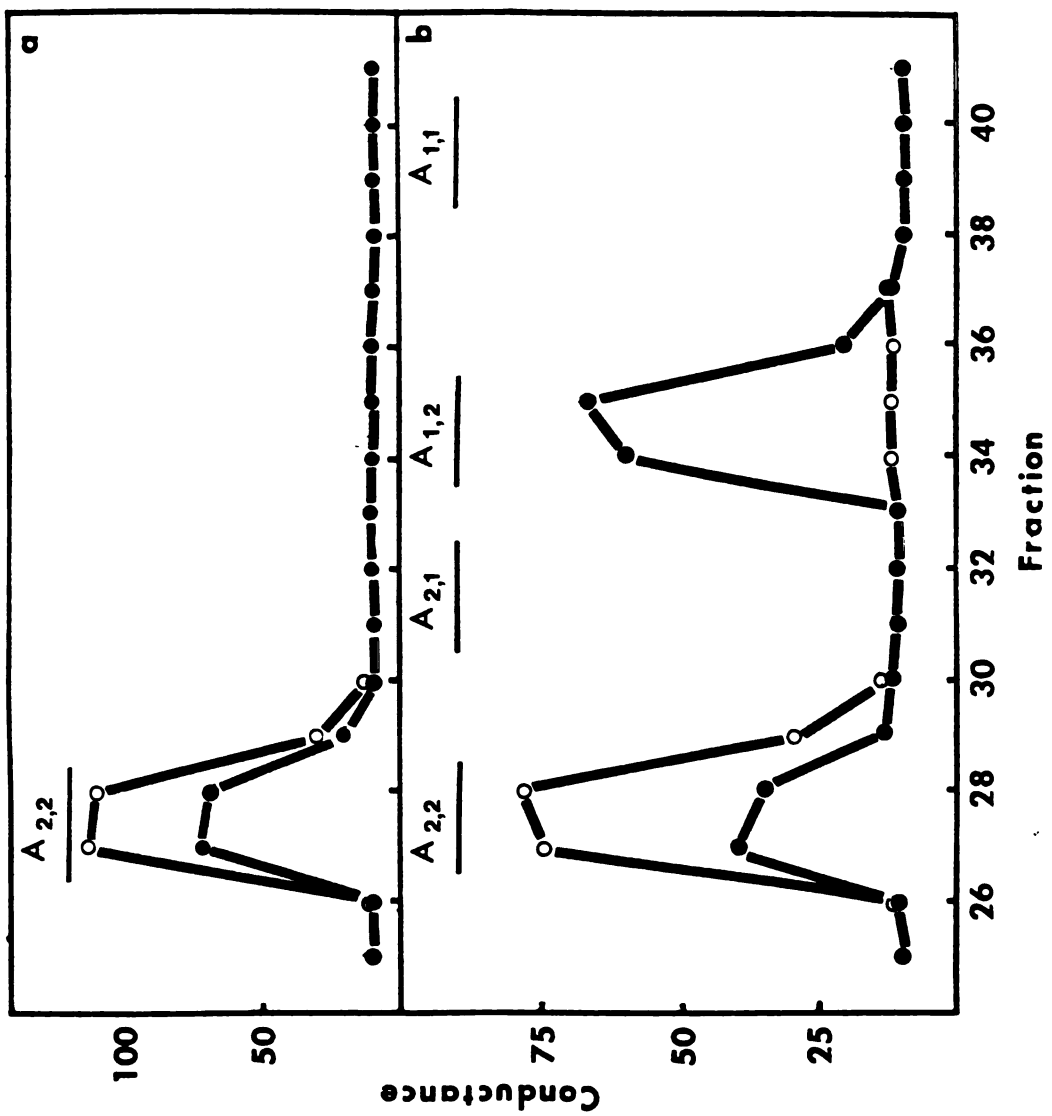
Biological activity of the toxoids. We previously reported that the natural mixture of toxoid III isomers induced loss of electrolytes and produced runner lesions on some but not all clones of sugarcane that are sensitive to the 4-gal toxin molecule (9). The 5 clones of sugarcane used in this experiment were highly susceptible to the pathogen and

sensitive to the 3 forms of toxin. Each form of purified toxin was partially digested, to identify the toxic moiety in the toxoid III preparation. The resulting mixtures of toxin and toxoids were separated with reverse phase HPLC (20% CH_3CH, 2 ml/min) and fractions (1 ml) were collected. Aliquots taken from each fraction were bioassayed on sugarcane clones Co 453 and NG 77-234.

The digests of toxins B and C contained no toxoids that caused damage to any of the five clones; only those fractions containing undigested toxin (4 gal units/mol) induced loss of electrolytes. In the partial digest of toxin A, aliquots of fractions containing the toxin (4 gal/mol) caused loss of electrolytes from clones NG 77-234 and Co 453 (Fig. 6). No other fractions induced electrolyte loss from Co 453. Those aliquots taken from fractions containing toxoid $A_{1,2}$ (but not from any other toxoid-containing fractions) induced electrolyte loss from clone NG 77-234 but not from Co 453. As a control, undigested preparations of each toxin were chromatographed as above, and as expected only fractions containing the toxins (4 gal/mol) induced electrolyte loss. This confirms that toxoid $A_{1,2}$, produced by the action of β-galactofuranosidase on toxin A, is the toxic moiety in the toxoid III mixture of isomers.

Toxoid $A_{1,2}$, purified from culture fluids of H. sacchari, was tested for ability to induce electrolyte loss from several clones of sugarcane. Toxoid $A_{1,2}$ was toxic to clones NG 77-82, NG 77-234, and CP 73-1000 but was not toxic to Co 453 and NG 77-103. The 5 non-toxic isomers of toxoid III can act as inhibitors of $A_{1,2}$ (data not shown). The electrolyte loss-inducing ability of toxoid $A_{1,2}$ was compared to its parent molecule (toxin A) and to toxin C, the most active form of toxin. Although toxin A was less active than was toxin C, the removal of the left terminal

Fig. 6. Bioassay of toxoids resulting from partial digestion of toxin A. The toxoids and undigested toxin were separated by HPLC and fractions (1 ml) were collected. Aliquots (10 µl) of the fractions were assayed with sugarcane clone Co 453 (○) and NG 77-234 (●). (a) Eluate from digested preparation. (b) A preparation identical to that chromatographed in (a) was partially digested and the resulting toxoids were separated by HPLC. The bars (i.e., $A_{2,2}$) indicate the peaks that were present in each chromatogram. The solvent was 20% acetonitrile in water and the flow rate was 2 ml/min.


a
b
$A_{2,2}$
$A_{2,1}$
$A_{1,2}$
$A_{1,1}$
Conductance
100
50
75
25
26
28
30
32
34
36
38
40
Fraction

galactose unit resulted in a compound ($A_{1,2}$) which was about as active on the three sensitive clones as is toxin C (Table 2).

Protective effects of toxoids. It is apparent that the positions of galactose and the arrangement of double bonds in the sesquiterpene core are very important in determining toxicity. Do these structural characteristics play a significant role in determining how effective a toxoid will be at protecting tissues against toxin? In our previous examination of the protective abilities of the natural mixtures of toxoids we omitted one group of toxoid II isomers (9). This natural mixture of toxoids, designated II', contains the isomers $A_{1,1}$, $B_{1,1}$, and $C_{1,1}$. They elute from the LH-20 column as a single peak that is separate from the peak containing the other 6 isomers of the toxoid II group (Fig. 1).

The protective abilities of the natural mixture of toxoids II', II and I were compared using the standard protection bioassay (Table 3). Toxoid II' required four-fold higher concentrations than did toxoid II to give equal protection. Although all toxoids II and II' contain 2 galactose units, their arrangement plays a significant role in their effectiveness as inhibitors of toxin. Toxoid I, with only one galactose, provided significantly less protection than did toxoid II'.

The six isomers of toxoid III were tested for their protective ability on 2 clones of sugarcane (Table 4). As a control, each toxoid was again tested for ability to induce electrolyte loss on both sugarcane clones. Only toxoid $A_{1,2}$ induced loss of electrolytes from clone NG 77-234; no toxoid isomer induced losses from NG 77-103. In general, the toxoids protected against toxin C more effectively on clone NG 77-103 than on any other clone tested. Toxoid $C_{2,1}$ was consistently more effective than was $C_{1,2}$ or any other toxoid at protecting against

Table 2. Electrolyte leakage from sugarcane clone NG77-234 induced by toxins ($A_{2,2}$ and $C_{2,2}$) and toxoid $A_{1,2}$. Leaf disks were exposed to test solutions for 0.5 hr, and rinsed thoroughly. Conductance values were taken after 3 hr incubation in leaching solution.

Concentration (μM)	Losses induced by		
	$A_{1,2}$ (μmhos)	$A_{2,2}$ (μmhos)	$C_{2,2}$ (μmhos)
0.1	30.	4.	35.
1.0	109.	82.	116.
10.0	142.	136.	134.

Table 3. Comparative abilities of three natural mixtures of toxoid isomers to protect sugarcane tissues (clone NG 77-234) against toxin C. Toxoid I is a natural mixture of isomers with one galactose unit. Toxoid II' is a natural mixture of the isomers $A_{1,1}$, $B_{1,1}$ and $C_{1,1}$. Toxoid II is the natural mixture of isomers with galactose arranged 2,0 and 0,2. Leaf disks of clone NG 77-234 were exposed to toxin and toxoids simultaneously for 0.5 hr, rinsed, and incubated in the leaching solution for 3 hr, when conductance was determined.

Toxoid Concentration (μM)	Losses induced by toxin-C (0.75 μm) in the presence of toxoid		
	I	II'	II
	(μmhos)	(μmhos)	(μmhos)
0	127	127	127
50	-[a]	-	52
100	120	85	-
200	103	53	-
400	95	30	-

[a]Not determined.

toxin C. In general the toxoids with the A sesquiterpene were slightly less effective than were those with the B sesquiterpene (Table 4). The arrangement of the gal units made little or no difference with toxoids containing the A or B sesquiterpene, but made a significant difference for toxoids containing the C sesquiterpene.

The effectiveness of toxoids $C_{2,1}$ and $C_{1,2}$ at protecting against toxin C were tested at several toxoid concentrations. Toxoid $C_{2,1}$ was more effective than was $C_{1,2}$ at protecting leaf tissue against toxin C-induced loss of electrolytes at each of several toxoid concentration (Table 5).

It was difficult to obtain purified preparations of the toxoid II isomers. Those toxoids with the A or B sesquiterpene were purified from the culture fluids, which had a very low amount of $A_{0,2}$ and $B_{0,2}$ isomers. Thus, only $A_{2,0}$ and $B_{2,0}$ were obtained in amounts required for protection assays. Toxoids containing the C sesquiterpene were purified from a partial digest of toxin C. The protective effectiveness of these four toxoid II isomers were tested, using the standard protection bioassay with three clones of sugarcane (Table 6). Toxoid $C_{0,2}$ was consistently more effective than was $C_{2,0}$ at protecting tissues against toxin C. $B_{2,0}$ and $C_{2,0}$ were equal in effectiveness, but toxoid $A_{2,0}$ was nearly as effective as was $C_{0,2}$.

DISCUSSION

Three different isomers of HS toxin were found in preparations purified by a previously reported procedure (5). These forms of toxin were reported to differ in the sesquiterpene core (12). Each form was toxic only to sugarcane susceptible to the pathogen, with no effect on

Table 4. Comparative abilities of the six isomers of toxoid III to protect sugarcane tissues (clones NG 77-103 and NG 77-234) against toxin C. The treating solution contained toxin C at 0.75 μm without (none) or with toxoids at 50 μm. The standard protection bioassay was used.

Toxoid preparation	Toxin-induced losses from clones	
	NG77-234	NG 77-103
	(μmhos)	(μmhos)
III mix[a]	39	3
$A_{2,1}$	36	12
$A_{1,2}$	-	13
$B_{2,1}$	21	4
$B_{1,2}$	32	6
$C_{2,1}$	8	0
$C_{1,2}$	38	6
None	116	87

[a]The natural mixture of all toxoid III isomers.

Table 5. Comparative abilities of toxoids containing sesquiterpene C in protecting sugarcane tissues (clones Co 453 and NG 77-234) against toxin C. The treating solution contained toxin C at 1.0 μm without or with toxoid at the indicated concentrations. The standard protection bioassay was used.

Toxoid concentration (μM)	Toxin-induced losses in the presence of toxoid			
	Clone Co 453		Clone NG 77-234	
	$C_{2,1}$ (μmhos)	$C_{1,2}$ (μmhos)	$C_{2,1}$ (μmhos)	$C_{1,2}$ (μmhos)
50	3	7	16	66
25	13	27	58	102
10	44	67	106	112
0	81	81	110	110

Table 6. Comparative abilities of four isomers of toxoid II to protect sugarcane tissues (clones NG 77-103, NG 77-234 and Co 453) against toxin C. The treating solution contained toxin C at 0.75 μm (for clones NG 77-103 and NG 77-234) and at 1.0 μm (for cone Co 453), without (none) or with toxoids at 50 μm. The standard protection bioassay was used.

	Toxin C-induced electrolyte losses from clones		
Toxoid preparations	NG77-103	NG77-234	Co 453
	(μmhos)	(μmhos)	(μmhos)
II mix[a]	14	34	35
$A_{2,0}$	20	49	41
$B_{2,0}$	34	61	58
$C_{2,0}$	30	59	56
$C_{0,2}$	18	44	34
None	87	72	76

[a]The natural mixture of all toxoid II isomers with galactose arranged 2,0 or 0,2.

sugarcane resistant to the pathogen. The relative toxicity of each form of toxin was tested and compared. Each toxin isomer produced runner lesions on susceptible sugarcane and each caused electrolyte leakage from all susceptible clones that were tested. However, the three forms differed in relative severity of induced losses. Apparently, differences in the position of a double bond in the sesquiterpene core of toxin, which results in differences in shape of the toxin mol, has a major role in determining how active the toxins are at inducing electrolyte loss.

The HS toxin molecule apparently has two chains of two galactose units each, attached to an unsymmetrical sesquiterpene core (12). This arrangement of gal has been designated 2,2 (12). Previous work has shown that the toxoids can be produced by enzymatically removing 1, 2, or 3 units of galactose from toxin. The earlier study was done with a mixture of the three toxin isomers (7). We have now partially hydrolyzed each isomer of toxin and identified the seven resulting toxoids. Two isomers of toxoid III were formed from each isomer of toxin. This is consistent with a galactose arrangement pattern in the toxins of 1,3; 3,1; or 2,2. It is not consistent with an 0,4 or 4,0 arrangement, which would produce only 1 form of toxoid III. Further, three isomers of toxoid II were found to be produced from each isomer of toxin. This rules out a linkage of 1,3 or 3,1, which would result in only 2 forms of toxoid II. The toxin must therefore have a linkage of 2,2. The existence of three isomers of the sesquiterpene results in toxins $A_{2,2}$, $B_{2,2}$, $C_{2,2}$.

We reported previously that a mixture of toxoid III isomers was toxic to some sugarcane clones, but not to others. Data reported here show that of these isomers, only $A_{1,2}$ induced electrolyte losses and produced a runner lesion. It is interesting to note that this toxoid was

produced by removal of the left terminal galactose unit from toxin A, the least active form of toxin. Toxoid $A_{1,2}$ is just as active at inducing electrolyte losses in certain sugarcane clones as is toxin C, the most active form of toxin. Furthermore, the fact that only some susceptible clones of sugarcane are sensitive to toxoid $A_{1,2}$, whereas many other clones are sensitive to the toxins (4 gal/mol), suggests that the mode of toxicity for toxin and toxoids may be complex. Possibly the putative receptor proteins of sugarcane differ slightly, or the mechanism of leakage differs from clone to clone.

The data indicate that the sesquiterpene isomer and the number and arrangement of galactose units is very important in determining toxicity of a compound. Are these factors significant in determining whether or not a toxoid will effectively prevent action of toxin? We previously reported that the natural mixture of toxoids with 3 gal units protected better than those with two gal units (9). Each of the six toxoid III isomers was tested for effectiveness at protecting against toxin C. Toxoid $C_{2,1}$ protected much better than did toxoid $C_{1,2}$. Apparently the location of galactose units is important in protective ability. The arrangement of galactose units in the toxoids containing sesquiterpenes A and B did not make as much different as did the arrangement in toxoids containing the C sesquiterpene. In general, toxoids with the B sesquiterpene protected better than did those with the A sesquiterpene. It will be interesting to use toxin B to determine whether or not the B sesquiterpene toxoids will protect better than will those with the C sesquiterpene.

Only seven isomers of toxoid II were obtained in amounts necessary for protection assays. Toxoids with the 1,1 arrangement did not protect

as well as did toxoids with the 2,0 or 0,2 arrangements, even though all three contain 2 gal units/molecule. Apparently a two galactose chain is an important factor in determining how effective a toxoid will be at inhibiting the action of the toxin. Toxoid $C_{0,2}$ was more effective than was $C_{2,0}$ at protecting against toxin C. This is in contrast to the results with toxoid III showing that $C_{2,1}$ was more effective than was $C_{1,2}$. The other toxoid II isomers differed in effectiveness, but none was as effective as was any of the isomers of toxoid III at preventing toxin C induced electrolyte losses from sugarcane tissue.

Are our data compatible with the hypothesis that toxin binds to and changes a specific protein found in sensitive sugarcane clones? The data on protective effects of toxoids appear to be compatible with such a hypothesis. It is unlikely that toxin and toxoids are interacting with each other. The simplest explanation of protective effects of toxoids is that they interact with a toxin receptor, thus reducing toxin interaction with the receptor. The toxoids are not toxic; thus their interaction with a receptor apparently is not sufficient to result in electrolyte loss. The interaction of a receptor with toxin may be qualitatively different from the interaction of a receptor with a toxoid. Perhaps only the toxin, because of its specific shape, can cause a conformational change in the receptor which can result in electrolyte loss. These are tentative suggestions, because no kinetic data on toxin uptake or action are available. Whole tissue does not provide sufficiently precise kinetic data to determine whether or not the protective effects of toxoids are competitive. Better assays will be required; among the possibilities are use of cell cultures or protoplasts, which should be uniform host-cell populations. Eventually, cell-free preparations of

receptor proteins and radioactively-labeled toxin and toxoids will be required for a firm conclusion regarding toxic action.

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GENERAL DISCUSSION

Helminthosporium sacchari was shown in 1971 (4) to produce a highly active toxin that is selectively toxic to sugarcane clones (cultivars) that are susceptible to the fungus. Soon after this discovery was published, a structure of the toxin was presented (5). This work initiated Dr. Strobel's very active research program which included studies on mode of toxic action and resistance to toxin (7). The work attracted much attention, and became the most-cited model for the molecular basis of plant disease development.

My dissertation research began as a reexamination of the chemical characteristics of the selective toxin from H. sacchari. Improved procedures were developed to isolate and purify the toxin (section I and IV). Characterization by NMR, MS, derivatization, and other techniques showed clearly that the earlier description of toxin (5) was incorrect. The toxin contains four units of galactose (not one as originally described); the galactose is in the relatively rare furanose form, with β linkages. The core of toxin is a sesquiterpene, not cyclopropanol as first described. This part of the dissertation has been published (section I).

Several analogs of toxin which contain three, two, or one units of galactose were found in cultures of H. sacchari. These compounds might be precursors or degradation products of toxin. They were called toxoids, for convenience, because they are analogs of toxin that protect

susceptible tissues against toxin. Another significant finding was that H. sacchari produces an enzyme (β-galactofuranosidase) which removes galactose units from the toxin molecule, forming toxoids. The kinetics of the production of β-galactofuranosidase, toxin, and toxoids indicated that the toxoids are degradation products of toxin. It is interesting that the β-galactofuranosidase activity in H. sacchari cultures is very low when compared to the activities of four other species of Helminthosporium. If the β-galactofuranosidase activity was as high in liquid cultures of H. sacchari as it is in cultures of other species, then very little toxin would accumulate. It is only because HS toxin can be obtained at up to 200 mg/liter of culture fluids that much of this work could be done.

When HPLC became available to the laboratory, I found that my preparations of toxin and toxoids were a mixture of isomers (section IV). Three forms of HS toxin were separated with reverse phase HPLC. Each isomer of HS toxin was toxic to susceptible but not to resistance clones of sugarcane, although each differed from the others in relative ability to induce loss of electrolytes from susceptible sugarcane tissues. Using a β-galactofuranosidase, each isomer of HS toxin and of toxoid III was partially hydrolyzed and the resultant toxoids were separated and identified with HPLC. This information allowed me to identify the sesquiterpene isomer and the number and arrangement of galactose units in the toxins and toxoids. The work also confirms other reports (2) that toxin contains two chains of β-1,5 linked galactofuranose units.

The toxoids were isolated by HPLC and tested for biological activity (section IV). An assay based on measurement of the galactose released by acid hydrolysis of these compounds was developed to measure small amounts

of the toxin and toxoids. Only the three isomers of toxin and one isomer of toxoid III are toxic to sugarcane; the toxoid III isomer was toxic to three but not to two other clones of sugarcane that were all susceptible to the pathogen and sensitive to the toxin (four galactose units). It is interesting to note that this isomer of toxoid III was more toxic than was the toxin from which it was derived. The other five isomers of toxoid III and the toxoids with two or one units of galactose were not toxic to any of the sugarcane clones tested.

Each of the six isomers of toxoid III and seven of the toxoid II isomers were tested for ability to protect sugarcane tissue from toxin-induced damage. The number of galactose units in each toxoid was the most important factor in determining how effective a toxoid was at protection. Toxoids that contained three units of galactose protected better than did any of the toxoid II isomers. Toxoid I provided much less protection than did the toxoids that contained two units of galactose. These and other results indicated that the sesquiterpene isomer and the number and arrangement of galactose units are all important in determining how effective a compound will be at inducing or preventing toxin-induced loss of electrolytes from sugarcane tissue (section III and IV).

The current hypothesis for explaining the high degree of specificity which is characteristic of HS toxin involves a receptor protein (7). Many data have been published to establish this hypothesis but the work has been severely criticized (1). The hypothesis states that a protein in sensitive tissues binds toxin whereas a similar but different protein in insensitive tissue is unable to bind toxin. Binding was said to cause a change in the shape of the receptor protein. The change in some way

allows the pathogen to colonize the host tissue. Are the data presented in this dissertation compatible with the receptor protein hypothesis. If toxin is interacting with a receptor protein, then the toxoids may also interact with this receptor. However, a simple interaction may not be sufficient to cause tissue damage. Toxoids are not toxic even at high concentrations (100 μM). Toxin may be unique because it cannot only interact with the receptor but its binding may change the shape of the protein enough to alter its function. Toxoids could bind to a limited degree but may not induce sufficient change in shape of the protein to exert a toxic effect. These considerations indicate that my data could be compatible with a protein receptor model.

My aim was to contribute to an understanding of plant disease development at the molecular level. However, the full significance of the work will require as a first step a quantitative determination of toxin and toxoid production in infected tissue. Toxin production at the site of initial colonization has not been shown, although toxin is clearly being produced in older lesions as evidenced by the development of runner lesions and by isolation of toxin from these lesions (6). Preliminary studies of the toxin produced by H. sacchari fit the pattern of a host selective toxin yet only suggest that the toxin is necessary for disease development. Studies such as those done with H. carbonum and H. victoriae are needed for H. sacchari (3). The production of radio-labeled toxin and attempts to isolate a receptor protein also should have a high priority.

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