Plant Physiol. (1983) 72, 530–534
0032-0889/83/72/0530/05/$00.50/0

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

Received for publication November 8, 1982 and in revised form January 25, 1983

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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

[1] Supported in part by the National Science Foundation (grant no. PCM-8100711). Michigan Agricultural Experiment Station Journal Article No. 10784.

[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.

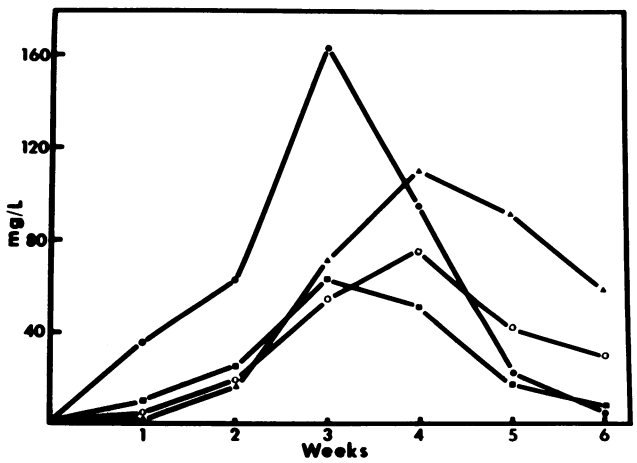

FIG. 1. Concentrations of toxin (●), toxoid III (■), toxoid II (○), and toxoid I (▲) in culture filtrates of *H. sacchari*, as determined by quantitative GLC.

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).

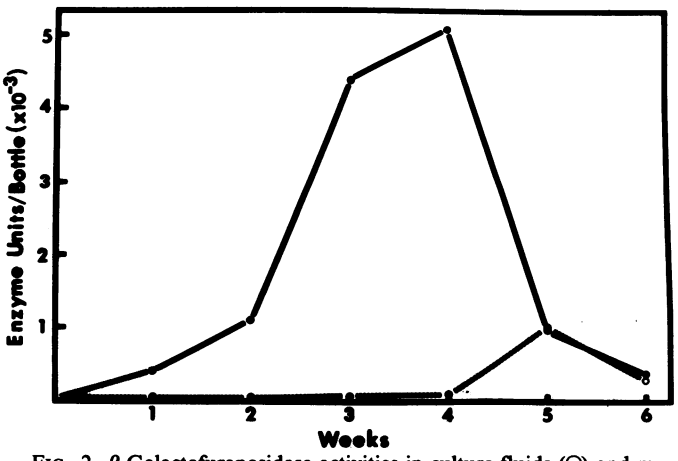

FIG. 2. β-Galactofuranosidase activities in culture fluids (○) and mycelium (●) of *H. sacchari* over a 6-week period. Cultures were grown in Roux bottles, each containing 200 ml of medium.

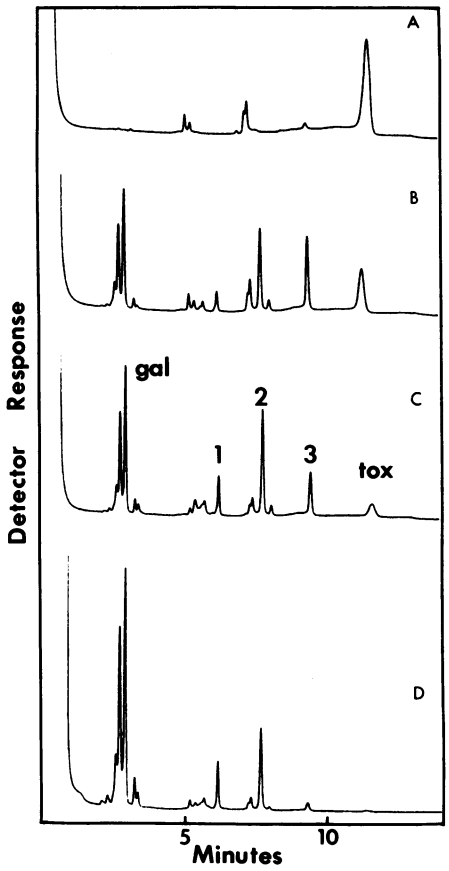

FIG. 3. GLC of products resulting from enzyme hydrolysis of HS toxin. Products are galactose (gal) and toxoids I, II, and III (1, 2, and 3, respectively). Determinations were made with trimethylsilyl derivatives, using a temperature regime of 130°C for 1.0 min followed by increases of 30°C/min up to 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.

Acknowledgments—We wish to thank the following people for their help: Ann Hancock for expert technical assistance; J. E. Gander, Department of Biochemistry, University of Minnesota, for samples of β-galactofuranosidase; Jack C. Comstock (Hawaii Sugar Planters), Jack Dean (USDA, Canal Point, FL), and Owen Sturgess (Bureau of Sugar Experiment Station, Indooroopilly, Queensland, Australia) for cultures of *H. sacchari*.

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