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Biochemical Basis of the Resistance of Sugarcane to Eyespot Disease

(helminthosporoside/binding protein/membranes)

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ABSTRACT **Helminthosporoside is the host-specific toxin produced by *Helminthosporium sacchari*, the organism causing eyespot disease on sugarcane. Clones of sugarcane susceptible to the toxin possess a membrane protein that binds the toxin. Membranes of resistant clones do not bind the toxin. In this study, a binding protein from a susceptible clone was compared with its counterpart from a resistant clone. The protein from the resistant clone did not bind the toxin unless it was first treated with mild detergent. The two proteins are antigenically identical, have the same molecular weight, and each contains four subunits. They differ slightly in their electrophoretic mobility and vary with respect to four different aminoacid residues. The basis of resistance of clone H50-7209 to eyespot disease is directly associated with the structurally altered membrane-binding protein.**

The causal agent of eyespot disease of sugarcane is *Helminthosporium sacchari* (Van Breda de Haan), which occurs in most of the cane-growing areas of the world. The fungus infects the leaves and stems of sugarcane, after which an eyespot lesion is produced. A reddish streak or "runner" develops upwardly from the point of infection and does not harbor the fungus. Runner formation is the entire result of a toxin produced by the fungus (1). Furthermore, the toxin is host specific, in that it affects only those clones of sugarcane that are susceptible to the fungus. A direct correlation between the degree of susceptibility to the fungus and reaction to the toxin in several clones of sugarcane was established by Steiner and Byther (1). Steiner and Strobel (2) isolated the host-specific toxin from *H. sacchari*, identified it as 2-hydroxycyclopropyl-α-D-galactopyranoside, and designated it helminthosporoside. Further, Strobel and Steiner (3) showed that helminthosporoside was present in the runners on naturally infected sugarcane and in quantities large enough to cause symptoms.

Strobel *et al.* (4, 5) postulated that the toxin affected cellular membranes accounting for the initial water-soaking symptoms expressed in toxin-treated tissues. One of the first ultrastructural changes observed in treated tissues was alterations in chloroplast membranes. Strobel (5) established that the host specificity of the toxin resides with a toxin-binding protein localized in membranes of those clones that are susceptible to the toxin. The membranes of resistant clones do not bind the toxin, whereas those clones giving an intermediate reaction to the toxin likewise bind only intermediate amounts of the toxin relative to the membranes of the susceptible clones. The binding-protein from the membranes of a susceptible clone was purified and shown to have a molecular weight of 48,000 (5). It has four subunits and at least two binding sites for the toxin.

Inasmuch as membrane preparations from a group of resistant clones did not possess binding activity for helminthosporoside, it was of interest to examine them for proteins comparable to that of the toxin-binding protein of the susceptible clone, the idea being that herein may reside the basis for toxin resistance and hence resistance to this important disease of sugarcane. Thus, this report shows the comparative binding activities, and the chemical and immunological properties of the toxin-binding protein from a susceptible and a resistant clone of sugarcane.

MATERIALS AND METHODS

Sugarcane. The clones of sugarcane, 51 NG 97 (susceptible) and H50-7209 (resistant), were obtained from Dr. R. Coleman, United States Department of Agriculture, Beltsville, Md. The stalks were planted in large plastic pots and grown at 22 ± 5° under greenhouse conditions.

Binding Assays. Equilibrium dialysis was performed on a Kontron Diapack equilibrium dialyzer run at 24 rpm at 30° for 8 hr. All determinations were done on 0.5 ml of the protein or membrane preparations in half of a dialysis cell. The other half cell contained 0.14 μmol of helminthosporoside of a specific activity of 7.4 nCi/μmol made up to 0.5 ml with 0.01 M Tris·HCl buffer (pH 7.2). The labeled helminthosporoside was prepared as described (5). In assays in which the purified binding protein was used, the amount of helminthosporoside bound was calculated by determining the difference in radioactivity between half cells. When membrane preparations were used, the amount of toxin bound was determined as described by Strobel (5) in that the radioactivity was determined in one half cell only.

Preparation of Binding Protein and Membranes. The techniques used to acquire membranes and the binding proteins were identical to those of Strobel (5). Since no binding assay could be used to follow the protein purification of the protein from the resistant clone, the same techniques with a Bio-Gel P-100 column were applied. Ultimately the contents of those tubes with the same elution volume as the protein from the susceptible clone were pooled and concentrated. Protein was determined by the method of Lowry *et al.* (6).

Immunological Studies. An antiserum to the binding-protein from the susceptible clone was prepared by injecting the thigh muscles of each of two rabbits with 2 ml of a solution made by mixing 2 ml of a solution containing 0.85% NaCl, 0.5 mg of purified binding protein, and 12 mg of Tris·HCl buffer (pH 7.2) with 2 ml of Freund's complete adjuvant. Weekly injections over a 4-week period were administered to the rabbits. Only the first two injection solutions contained the adjuvant. Rabbits were bled by the ear-wound method

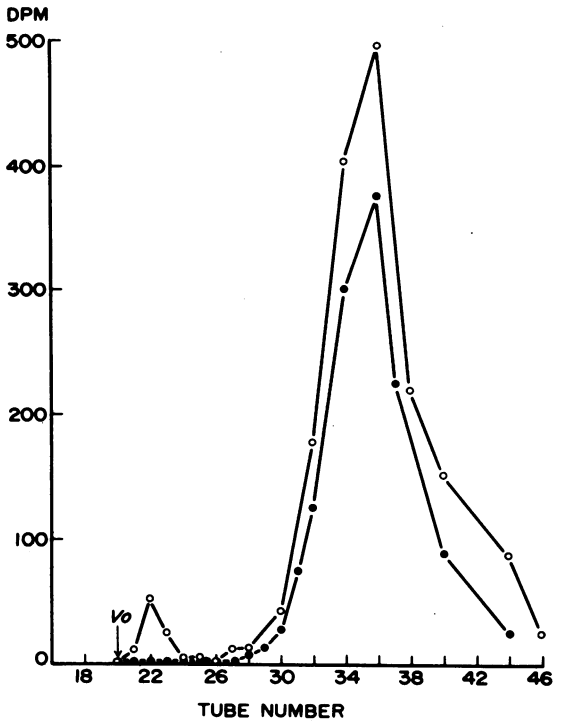

FIG. 1. Binding of [^{14}C]helminthosporoside by the aged purified binding protein of disease-resistant sugarcane clone H50-7209 (O——O), and also the freshly prepared binding protein (●——●). The protein (170 μg) was incubated with helminthosporoside and then applied to a column of Sephadex G-25 and eluted with 50 mM Tris·HCl buffer (pH 7.2). Aliquots of 1 ml were collected, and radioactivity was determined in each tube. The void volume is at tube 20.

3 weeks after the last injection. Serum was obtained by centrifugation of the blood after it congealed at room temperature followed by incubation at 4° for at least 12 hr. To test for antigen–antibody reactions, the double-diffusion method of Ouchterlony (7) was used, with 1% agarose made 75 mM (pH 8.6) with respect to veronal buffer. The center-well depot contained undiluted antiserum and the antigen depots contained about 40 μg of purified binding protein. The plates were incubated in a moist chamber for 24 hr at room temperature (24°) and then placed at 4° for 1–2 days.

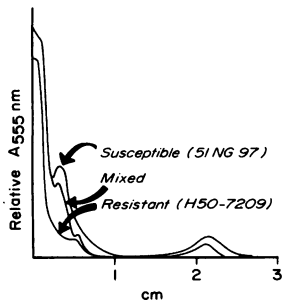

FIG. 2. Densitometer tracings of the binding proteins, separate and mixed, from clones H50-7209 and 51 NG 97. Electrophoresis was conducted on acrylamide gels for 1.5 hr. The gels were stained with Coomassie blue, destained, and then examined on a Joyce Lobel Densitometer at 555 nm. The peaks for the binding proteins are at 0.5 cm for the one from H50-7209 and 0.33 cm for the one from 51 NG 97.

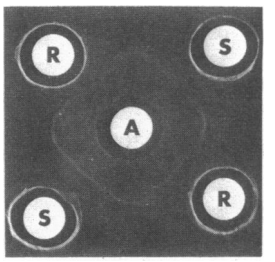

FIG. 3. Immunodiffusion pattern of the purified binding proteins from both the susceptible and the resistant clones. The outside wells contained purified binding protein from the resistant clone (*R*) and the susceptible clone (*S*), and the center well contained antiserum (*A*) prepared to the purified binding protein from the susceptible clone.

Electrophoresis. Disc gel electrophoresis was performed on 7.5% acrylamide gels at pH 8.5 by the procedures of Davis (8). Sodium dodecyl sulfate gels were run at pH 7.2, and molecular weights of the proteins were estimated by the procedures of Weber and Osborn (9). All gels were stained for 2 hr with 0.1% coomassie blue and destained in an aqueous solution of 7.5% acetic acid containing 5% methanol.

Aminoacid Analysis. Samples from the Bio-Gel column were prepared for hydrolysis by shaking them with 2–3 equal volumes of chloroform to remove the detergent. The precipitated protein was then dialyzed against methanol–water 4:1 (v/v), followed by dialysis against distilled water. The precipitated protein was washed with several rinses of distilled water after it was pelleted by centrifugation. The protein was subjected to hydrolysis in 1 ml of constant boiling HCl in an evacuated tube for 24 hr at 120°. Aminoacid analysis were performed on a Beckman model 120 E aminoacid analyzer.

Radioactivity Determination. All aqueous samples (0.5–2.0 ml) were mixed with 14 ml of Aquasol (New England Nuclear Corp.) before counting. Radioactivity measurements were made on a Packard Liquid Scintillation Spectrometer model 3360 and counts were corrected to disintegrations per min with the use of a linear quench curve.

RESULTS

Binding Experiments. The membrane preparation of the resistant clone H50-7209 did not bind helminthosporoside, whereas the membrane preparation from the susceptible clone 51 NG 97 effectively bound the toxin (5). Treatment of the membrane preparation with 1% Triton X-100 in 50 mM Tris·HCl buffer (pH 7.2) for 4 hr at 4° followed by centrifugation at 50,000 × *g* resulted in solubilized membrane proteins in the supernatant liquid. This preparation from the susceptible clone bound the toxin, but there was no binding activity in the comparable preparation from the resistant clone (Table 1). A purified membrane protein from the resistant clone with the same elution volume from the Bio-Gel P-100 column as that of the susceptible clone was obtained, but it did not possess binding activity (Table 1). However, when this protein was aged for 3 weeks at 4° in 1% Triton X-100, or if it were incubated with 0.1% sodium dodecyl sulfate during the dialysis procedure, the protein ultimately bound helminthosporoside (Table 2).

An experiment was performed that confirmed that the binding of helminthosporoside occurred in a purified protein

TABLE 1. *Relative binding of helminthosporoside by membrane and protein preparations from a resistant clone (H50-7209) and a susceptible clone (51 NG 97) of sugarcane*

Preparation	Helminthosporoside bound (nmol/mg of protein)
Membranes from resistant clone (5.7 mg of protein per 0.5 ml)	0.0
Membranes from susceptible clone (4.3 mg of protein per 0.5 ml)	1.6
Crude membrane proteins from resistant clone (2.1 mg/0.5 ml)	0.0
*Crude membrane proteins from susceptible clone (2.1 mg/0.5 ml)	4.7
Pure binding protein from resistant clone	0.0
Pure binding protein from susceptible clone	61.7

* Membrane proteins were prepared by treatment of the membranes with 1.0% Triton X-100 for 4 hr at 4° followed by centrifugation to remove membrane debris.

preparation from the resistant clone that had been aged for 3 weeks in 1% Triton X-100. The protein from H50-7209 (170 μg) was incubated with 74 μg (5139 dpm) of helminthosporoside in 0.5 ml of 50 mM Tris·HCl buffer (pH 7.2) for 6 hr at 30°. The preparation was then passed through a Sephadex G-25 column (1.5 × 31 cm), and 1-ml fractions collected. The radioactivity retained near the void volume of the column was indicative that the protein was binding the toxin (Fig. 1). In a comparable experiment in which 170 μg of freshly prepared protein was used, there was no radioactivity retained at the void volume of the column (Fig. 1).

Electrophoretic Mobility. About 30 μg of purified binding protein from both the resistant and the susceptible clones of sugarcane were subjected to disc gel electrophoresis for 1.5 hr at 3 mA per tube. The results in Fig. 2 show that the electrophoretic mobilities of the two binding proteins were nearly identical, but the binding protein from the resistant plant had a slightly greater mobility. This result was confirmed by mixing the proteins from the two sources and then subjecting them to electrophoresis.

Immunological Crossreactivity. Gel double-diffusion experiments showed that the binding protein from the susceptible clone was antigenically identical to the protein from the resistant clone (Fig. 3) since there was no spurring of the bands. Since the antigen preparations contained several contaminating proteins in low concentrations (5), it was imperative to determine if the antigen in the antigen–antibody reaction was the binding protein. The problem was approached by purifying the binding protein from four sister acrylamide gels after one had been stained with coomassie blue to locate the protein. The region of the gels containing the binding protein was removed and placed in 0.05 ml of 10 mM Tris·HCl buffer (pH 7.2) at 4° overnight. The solution was removed and placed in a double-diffusion system with the antiserum. An antigen–antibody reaction was observed, confirming the fact that the binding protein was the antigen involved in the reaction with the rabbit antiserum. A normal serum gave no reaction with plant protein.

TABLE 2. *Binding of helminthosporoside by the binding protein of the resistant clone (H50-7209) subjected to various treatments*

Treatment*	Helminthosporoside bound (nmol/mg of protein)
None (fresh from column in 1% Triton X-100)	0.0
Incubation with 0.1% sodium dodecyl sulfate during dialysis	41.0
Incubation with 1% Triton X-100 for 3 weeks at 4°	60.0

* The amount of protein used in these experiments varied from 80–170 μg/0.5 ml of dialysis test solution.

Molecular Weight Estimates. The elution volume of the protein from the resistant clone from the 1.5 × 98 cm columns of Bio-Gel P-100 used to prepare that protein is identical to that of the binding protein from the susceptible clone (5). According to the standard curve prepared by Strobel (5), the estimated molecular weight of the protein from the resistant clone is 45,000–46,000. Furthermore, when the protein from the resistant clone was treated for 10 min in 0.2% sodium dodecylsulfate and then subjected to electrophoresis according to the procedures of Weber and Osborn (9), the protein had a molecular weight of 47,000–48,000 when compared to the mobilities of several standard proteins (5). However, when the protein was treated in 1% sodium dodecylsulfate for 8 hr and then subjected to electrophoresis, there was a major band showing a molecular weight of 12,000, which is com-

TABLE 3. *Aminoacid analysis of the binding protein from a resistant and a susceptible clone of sugarcane*

Amino acid	Residues per subunit*	
	Resistant clone H50-7209	Susceptible clone 51 NG 97
Lysine	8.7 (9)	7.8 (8)
Histidine	1.2 (1)	0.9 (1)
Arginine	4.4 (4)	4.2 (4)
Aspartic acid	10.3 (10)	10.0 (10)
Threonine	4.9 (5)	5.0 (5)
Serine	3.8 (4)	4.7 (5)
Glutamic acid	13.0 (13)	11.6 (12
Proline	4.9 (5)	4.9 (5)
Glycine	12.0 (12)	10.8 (11)
Alanine	9.5 (10)	9.6 (10)
Cysteine	0 (0)	0 (0)
Valine	7.5 (8)	7.8 (8)
Methionine	1.0 (1)	1.0 (1)
Isoleucine	5.0 (5)	5.3 (5
Leucine	9.5 (10)	9.7 (10)
Tyrosine	3.0 (3)	2.8 (3)
Phenylalanine	4.5 (5)	4.6 (5)

* All calculations are on the basis of 1 residue of methionine. The values in *parentheses* are the number of residues to the nearest integer.

parable to previous data presented by Strobel (5) on the binding protein from the susceptible clone, 51 NG 97.

Aminoacid Analysis. Aminoacid analysis of the protein from the resistant clone showed that it was virtually identical to the amino acids making up the protein from the susceptible clone (Table 3). However, a difference in one aminoacid residue was found in lysine, glutamate, serine, and glycine. The protein has the properties of a typical membrane protein in that it contains no cysteine and has an abundance of aliphatic amino acids.

DISCUSSION

The ability of the protein from the resistant clone to bind the toxin either after aging in Triton X-100 or treatment with 0.1% sodium dodecyl sulfate suggests that it too has a binding site or sites for helminthosporoside. The inability of the freshly prepared protein from the resistant clone to bind the toxin may be a hindrance to the binding sites imposed by the structure of the protein. Evidence that the protein from the resistant clone is structurally different from that of the protein from the susceptible clone is found in the aminoacid analysis data (Table 3) and the slightly different electrophoretic mobilities of the two proteins (Fig. 2). Nevertheless, these two proteins are immunologically identical, have relatively the same molecular weights, and are subunit proteins. It is suggested that the detergent treatment of the binding protein from the resistant clone allows for binding to occur as a result of some slight uncoiling of the subunit protein that then exposes the binding site. It is unlikely that the protein loses its quaternary structure by such detergent treatment since the loss of this structure presumably renders the protein inactive. This was suggested by Strobel (5) who showed that treatment of membranes of clone 51 NG 97 with 2 M urea rendered them unable to bind helminthosporoside, but removal of the urea by dialysis restored some binding activity.

No conclusive evidence has been presented that indicates the precise location of the binding protein in the cell. Most certainly it is a membrane protein, as pointed out by Strobel (5), who also suggested that it may be located in the plasma membrane. The nature of the toxin effect, namely water, sugar, and electrolyte leakage from treated leaves, could be explained on the basis of a change in the permeability of the plasma membrane. Strobel suggested that this change is initially brought about by a conformational change in the binding protein as it accepts the toxin causing a local phase transition in membrane lipids resulting in a permeability change of the membrane (5). After this, other membranes in the cell may then be affected, either by the toxin or degradative enzymes located in lysosomes (4).

It is interesting to speculate on the nature of the toxin-binding protein in clones of cane that are intermediate or irregular (5) in their reaction to the toxin. It seems possible that since sugarcane is genetically polyploid, and that the binding protein is a four subunit protein, that hybrid types of helminthosporoside-binding proteins may exist. This, of course, would happen by virtue of the nature of the DNA message for the subunit protein occurring at the same or different loci on a number of different chromosomes. It may also be that some clones of cane may contain both types of binding proteins as described in this report, but vary in amounts and/or locations in plant tissues.

The physiological importance of the toxin-binding protein to the cell has not been ascertained. It is conceivable that this protein represents a transport protein for normally occurring sugars or amino acids found in the cell sap. The binding protein in the resistant clone may represent a structurally altered protein conceivably arising from one or more mutations. This concept is not without precedent since Boos (10) recently presented convincing evidence for the presence of a structurally defective galactose-binding protein in a mutant strain of *Escherichia coli*. Furthermore, the mutant strain was successfully reverted to a galactose transport-positive strain that had a binding protein with the same properties as the wild-type protein. Nevertheless, it is difficult at this point to firmly indicate whether the protein from the resistant plant or the one from the susceptible plant represents the wild-type protein.

The logic applied to the relationship between the presence of a functional binding protein in a given clone and its relationship to disease resistance follows from the work of Steiner and Byther (1). These workers pointed out that the toxin reaction of 182 sugarcane clones was significantly correlated ($r = 0.88$) to their reaction to *H. sacchari*. Furthermore, because of this high correlation, large-scale screening for resistance can be accomplished accurately and rapidly with the use of helminthosporoside. Then, Strobel (5) correlated to the 0.05 level of significance the relationship between the reaction of 29 clones of sugarcane to helminthosporoside and the ability of a membrane preparation of any given clone to bind the toxin. He also showed that the binding site is a membrane protein. Thus, there exists a direct relationship between the ability of the fungus to cause disease by the toxin it produces and the presence in the host of an acceptor membrane protein for this toxin. Although there are undoubtedly other factors in the pathogen, such as degradative enzymes, that contribute to its ability to become established in the host, the principal factor responsible for runner symptom formation is helminthosporoside. Therefore, it follows that if the toxin does not have a site or sites in the host with which to interact, no symptoms will be produced. In the eyespot disease this appears to be the basis of resistance to the toxin that is ultimately related to disease resistance.

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