The Toxin-Binding Protein of Sugarcane, Its Role in the Plant and in Disease Development
(membranes/helminthosporoside/membrane protein interactions)

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ABSTRACT The toxin-binding protein of sugarcane susceptible to eyespot disease also possesses raffinose-binding activity. The Kₐ’s for binding are: helminthosporoside (toxin) 6.8 × 10⁻⁴ M, raffinose 2.5 × 10⁻³ M, and melibiose 2.6 × 10⁻⁴ M. Evidence obtained by administering [¹⁴C]raffinose to sugarcane protoplasts suggests that this protein participates in α-galactoside transport. Cells from a resistant clone of sugarcane do not possess an active-binding protein, and likewise, do not actively take up raffinose. Interestingly, the K⁺, Mg⁺⁺ ATPase (ATP phosphohydrolase, EC 3.6.1.3.) on the plasma membrane of the susceptible sugarcane is 30% activated by the toxin at 3 mM. In addition, toxin-treated tissue slices show a rapid uptake of [¹⁴Rb⁺]K⁺ which is in agreement with the toxin activation of the membrane K⁺, Mg⁺⁺ ATPase. Since the ATPase does not directly interact with the toxin, the activation effect occurs by means of the toxin-binding protein. Membrane proteins may be influenced by the toxin-binding protein acting by one of several different mechanisms.

The toxin-binding protein of sugarcane is associated with clones of sugarcane that are susceptible to eyespot disease caused by Helminthosporium sacchari (1). This protein is present on the plasma membrane of leaf cells (2) and binds the host-specific toxin, helminthosporoside, that is produced by H. sacchari (3). The toxin is produced in leaves naturally infected with H. sacchari (4). Although membranes prepared from leaves of sugarcane clones resistant to the toxin possess a protein comparable to the binding protein, they do not bind the toxin (5). The biochemical basis of resistance in cane to the eyespot disease is thus related to the host-specific toxin produced by the fungus and to the membrane protein in the plant that binds the toxin.

The binding protein consists of four presumably identical subunits each with a molecular weight of about 12,000 and it has at least two binding sites for the toxin (1). The normal physiological role of the binding protein in the plant and the detrimental physiological effects resulting from toxin-binding to the protein have not been previously established. This report presents evidence for the role of the toxin-binding protein in α-galactoside transport and describes how the toxin interferes with cellular functions by means of the binding protein.

MATERIALS AND METHODS

Sugarcane. The clones of sugarcane, 51 NG 97 (susceptible) and H50-7209 (resistant) were supplied by Dr. R. Coleman, USDA, Beltsville, Md. The stalks were planted in large plastic pots and grown at 22 + 5° under greenhouse conditions.

Protoplasts. Wall-less leaf cell protoplasts were prepared from both clones of sugarcane according to the procedures previously described (2).

Plasma Membranes. The basic procedure of Hodges et al. (6) was used to prepare the plasma membrane fraction of sugarcane leaves. This technique was used in a previous study on membranes of sugarcane and the recovery of plasma membranes was demonstrated (2). The discontinuous sucrose gradient procedure of Leonard and Hodges (7) was also effective in isolating the plasma membrane fraction from the 34 to 45% sucrose interface.

Toxin-Binding Protein. The toxin-binding protein was isolated from sugarcane leaf tissue as previously described by Strobel (1). The equilibrium dialysis technique was used to assay for toxin-binding activity and for α-galactoside binding activity (1).

ATPase. This activity was measured at 23° in a 0.5-ml volume containing 3 mM ATP (Tris or Na⁺ salt) at pH 7.2 with 10 mM Tris-HCl buffer and variable amounts of cations. The reaction was started by the addition of 300–500 μg membrane protein. The released P, was measured by the method of Lowry and Lopez (8). Protein was determined according to the procedure of Lowry et al. (9).

Substrates. [¹H]Helminthosporoside with a specific activity of 0.138 μCi/2.5 mg was obtained from cultures of H. sacchari to which were administered [¹H]sucrose (3). [¹⁴C]Raffinose, with a specific activity of 0.48 μCi/3.9 mg, was prepared from [¹⁴C]sucrose in the trans-galactosidase reaction described by Moreno and Cardini (10). [¹⁴C]Melibiose (0.12 μCi/0.97 mg) was obtained by subjecting a portion of [¹⁴C]raffinose to digestion with a purified preparation of sucase. [¹⁴C]Methyl-α-galactopyranoside (0.0039 μCi/2.75 mg) was prepared by refluxing [¹⁴C]methanol and α-α-galactopranoside over Dowex 50 (H⁺ form) for 18 hr according to the method of Bollenbach (11). The final product was chromatographed and detected as described below. The nuclear magnetic resonance spectrum of the synthetic compound, obtained on a Varian T-60 nuclear magnetic resonance spectrometer, was identical to authentic methyl α-galactopyranoside. No β form of the compound was detectable in the preparation.

¹⁴Rb Uptake Experiments. ¹⁴RbCl (0.00075 μmol) was mixed with 0.05 ml of 0.01 M KCl, and to this solution, was added a leaf disc of sugarcane (7 mg) that was 1 mm thick and 5–6 mm in diameter. ¹⁴Rb⁺ can be used as a measure of K⁺ uptake by plant tissues (12). The disappearance of ¹⁴Rb⁺
from the external medium was considered as the amount of K+.*Rb+ taken up by the leaf disc.

Chromatography. Chromatographic separation of sucrose, raffinose, and other sugars in protoplasts and protoplast suspensions was performed on Whatman no. 1 filter paper in a solvent of ethyl acetate–pyridine–H2O (6:4:3). Sugars were detected according to the procedures of Trevelyan et al. (13).

Counting Methods. 1H and 14C compounds in aqueous solution were placed in 12 ml of Aquasol (New England Nuclear Corp.) and counted in a Packard Liquid Scintillation Spectrometer. The 1H and 14C cpm were corrected to dpm with quench curves. Small volumes of aqueous samples including those from the *Rb experiments were counted in a standard counting solution (3).

Materials. 1H- and 1C-labeled compounds were supplied by Amersham/Searle. *RbCl was obtained from New England Nuclear Corp. Enzymes and other biochemicals were purchased from Sigma Chemical Co.

RESULTS

Previously, we (1) demonstrated that a number of common α-galactosides competitively inhibited the binding of helminthosporoside (2-hydroxycyclopropyl-α-D-galactopyranoside). This suggested that a possible physiological role of the binding protein may be in α-galactoside transport. Raffinose is an α-galactoside found in sugarcane and perhaps is involved in carbon skeleton transport. The purified binding protein (280 µg) from clone 51 NG 97 was incubated 2 hr with 0.145 µmol of [14C]raffinose in 0.2 ml of 0.36 M Tris·HCl buffer at pH 7.4. The preparation was chromatographed on a 0.5 X 44-cm column of Sephadex G-25 and 1-ml fractions collected. The results showed that the purified protein bound raffinose whereas after boiling, it did not (Fig. 1). Further, a comparable preparation from resistant clone H50-7209 (5) did not bind raffinose. The Kₐ of binding of a number of α-galactosides by the binding protein was determined (Table 1). Interestingly, the Kₐ for raffinose and melibiose binding approximated that of helminthosporoside binding, with a higher value for methyl α-galactopyranoside.

The binding protein was examined for other enzyme activities using standard procedures, but no α or β galactosidase activity, ATPase, or trans-galactosidase activity, was detected.

Protoplasts and α-galactosides

Inasmuch as the toxin-binding protein also had an affinity for common α-galactosides, its apparent involvement in raffinose uptake by protoplasts was examined. Approximately 10⁶ protoplasts, in 0.05 ml of 0.01 M K-citrate buffer at pH 5.6 and 0.3 M sucrose were incubated with [14C]raffinose (2.4 X 10⁻⁴ M).

The incubation was carried out at 23° in small V-shaped vials. Since the protoplasts are extremely fragile, the common filtration procedures usually used to follow metabolite uptake by bacteria were avoided. Instead, since viable protoplasts float to the surface of the incubation medium, a portion of the external medium taken from beneath the protoplast layer was periodically removed with a 10 µl Hamilton syringe and the radioactivity determined. After each sample was taken, the suspension of protoplasts was carefully stirred. Within 5 min, the protoplasts again began to form a layer on the incubation medium. Fig. 2 shows the uptake of [14C]raffinose by the protoplasts as a function of time. When the protoplasts were preincubated in 1.0 X 10⁻⁴ M helminthosporoside, there was no net uptake of raffinose over a 30-min period. Further, there was no net uptake of raffinose by protoplasts from the resistant clone H50-7209 that were not treated with the toxin (Fig. 2). This was expected since this clone does not possess a functional binding protein (Fig. 1). The apparent uptake of label that occurred within the first 15 min in all preparations seems to be a nonspecific binding phenomenon. In a comparable experiment, 30 min after incubation with labeled raffinose, the protoplasts were washed with 0.6 ml of 0.3 M glucose, citrate buffer solution, pelleted at 200 X g and placed in distilled H₂O. Under such conditions, rupture of the membrane occurred and the cellular contents were released. The aqueous solution was chromatographed and at

![Fig. 1. The binding of raffinose by 280 µg of toxin-binding protein of susceptible sugarcane clone 51 NG 97 (Ø) and by 280 µg of the binding protein counterpart from resistant clone H50-7209 (△). A boiled preparation of the protein from susceptible sugarcane yielded virtually the same curve as that from the resistant clone (Δ). Vo is the void volume of the column.](image)

![Fig. 2. Raffinose uptake by 3 X 10⁶ protoplasts of a susceptible clone of sugarcane (Ø) and a resistant (△) clone of sugarcane. Each preparation possessed 0.24 mg of membrane protein. The uptake of raffinose in susceptible protoplasts (Ø) after pretreatment with 1 mM helminthosporoside for 5 min.](image)

### Table 1. The Kₐ of binding of α-galactosides by the toxin-binding protein of sugarcane

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₐ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helminthosporoside</td>
<td>68</td>
</tr>
<tr>
<td>Raffinose</td>
<td>25</td>
</tr>
<tr>
<td>Melibiose</td>
<td>26</td>
</tr>
<tr>
<td>Methyl α-galactopyranoside</td>
<td>136</td>
</tr>
</tbody>
</table>

The binding protein was examined for other enzyme activities using standard procedures, but no α or β galactosidase activity, ATPase, or trans-galactosidase activity, was detected.
least 1.6 μmol of raffinose, and 0.09 μmol of sucrose were recovered. No labeling was present in glucose and fructose. The $K_m$ of raffinose uptake by protoplasts was approximately $9 \times 10^{-4}$ M.

Protoplasts from both clones were examined for uptake of [3H]helminthosporoside (Fig. 3). Incubation of $3 \times 10^4$ protoplasts in [3H]helminthosporoside (1.6 $\times 10^{-2}$ M) showed that protoplasts from the susceptible clone, took up the toxin whereas there was no net toxin uptake by protoplasts from the resistant clone. Experiments were conducted in which protoplasts of the susceptible clone pretreated with the toxin were washed and lysed in distilled H$_2$O. No [3H]helminthosporoside was recovered from the contents of the protoplasts indicating that the association of the toxin with the protoplasts is only at the outer surface and that transport of the toxin into the cell apparently does not occur.

**ATPase**

Sugar cane protoplasts susceptible to the toxin undergo a gradual swelling, distortion, and rupture similar to protoplasts in a hypotonic solution. Since the toxin did not cause an apparent build-up of raffinose in the cell, other factors leading to changes in cellular osmolarity were investigated. One consideration was the involvement of the toxin with the ATPase of the plasma membrane (7). In higher plants this enzyme is activated by K$^+$ and Mg$^{2+}$ and is presumably responsible for K$^+$ transport across the plasma membrane. Initially, it was established that the binding activities for raffinose and the toxin were indistinguishable. Further, this activity was distinct from the membrane ATPase activity (Fig. 4). This was accomplished by chromatographing the Triton X-100 treated membrane fraction through a Biogel P-100 column (Fig. 4) and assaying for these enzyme activities.

The plasma membrane fraction from sucrose density gradients was assayed for ATPase activity and shown to be activated by K$^+$ and Mg$^{2+}$ and only to a small extent by Ca$^{2+}$ (Table 2). When the membrane preparation was pre-incubated with 3 mM helminthosporoside there was a 30% increase in ATPase activity beyond that observed by cation activation. There was no toxin stimulation of ATPase activity in membranes from the resistant clone (data not shown).

**α-Galactosides**

Other than the toxin, did not possess this activation effect on the ATPase (Table 2). Treatment of the membranes with Triton X-100 abolished the toxin activation effect, but nevertheless, also increased the level of ATPase activity.

**DISCUSSION**

The protective effects of α-galactosides on symptom development after toxin treatment was strong presumptive evidence for the involvement of the toxin-binding protein as the primary site of toxin action (1). The results of the present work

![Figure 3](image-url)  
Fig. 3. [3H]Helminthosporoside uptake by $3 \times 10^4$ protoplasts of the susceptible (O) and the resistant (A) clone. Each preparation contained 0.24 mg of membrane protein.

![Figure 4](image-url)  
Fig. 4. Elution of ATPase activity (A), raffinose-binding protein activity (○), and protein (○), from a 1.5 × 95-cm column of Biogel P-100. The raffinose-binding activity eluted at the same volume as the toxin-binding protein activity (2). Vo is the void volume of the column.

**Table 2. Plasma membrane ATPase activity (susceptible clone 51 NG 97) as a function of the presence of various compounds**

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Reaction rate or nmol/mg of protein per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>8</td>
</tr>
<tr>
<td>ATP-Tris-HCl (1.5 mM)</td>
<td>50</td>
</tr>
<tr>
<td>ATP (1.5 mM) + CaCl$_2$ (1.5 mM)</td>
<td>60</td>
</tr>
<tr>
<td>ATP (1.5 mM) + KCl (50 mM)</td>
<td>80</td>
</tr>
<tr>
<td>ATP (1.5 mM) + MgCl$_2$ (1.5 mM)</td>
<td>98</td>
</tr>
<tr>
<td>ATP (1.5 mM) + KCl (50 mM) + MgCl$_2$ (1.5 mM) (complete)</td>
<td>104</td>
</tr>
<tr>
<td>*Helminthosporoside (3 mM)</td>
<td>134</td>
</tr>
<tr>
<td>*Helminthosporoside (1 mM)</td>
<td>112</td>
</tr>
<tr>
<td>*Raffinose (3 mM)</td>
<td>104</td>
</tr>
<tr>
<td>*Methyl-α-galactopyranoside (3 mM)</td>
<td>104</td>
</tr>
<tr>
<td>*Melibiose (3 mM)</td>
<td>104</td>
</tr>
<tr>
<td>*Triton X-100 (2.5 mg)</td>
<td>170</td>
</tr>
<tr>
<td>Triton X-100 (2.5 mg) + 3 mM helminthosporoside</td>
<td>170</td>
</tr>
</tbody>
</table>

* These compounds at the given concentrations were pre-incubated in 0.1 ml of the plasma membrane preparation 20 min prior to the ATPase assay in which the complete reaction mixture was used.

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**Table 2. Plasma membrane ATPase activity (susceptible clone 51 NG 97) as a function of the presence of various compounds**
support the concept that the toxin-binding protein has a normal physiological role in the \( \alpha \)-galactoside transport in cane tissues. This evidence is the following: (i) the similarity in kinetics of raffinose binding to the binding protein, and raffinose uptake by protoplasts; (ii) the lack of raffinose uptake by protoplasts from a resistant clone of cane that also lacks raffinose-binding protein activity; (iii) the complete inhibition of raffinose uptake by treatment of susceptible cane protoplasts with the toxin; (iv) the raffinose-binding activity was indistinguishable from the toxin-binding activity eluting from the Biogel P-100 column.

The evidence of active uptake of sugars by cane cells is in agreement with the results of Glassiou (14) and Maretski and Thom (15). Nevertheless, it does not seem possible for the total detrimental effect of the toxin to be entirely associated with \( \alpha \)-galactoside transport since cane cells resistant to the toxin behave normally without an apparent functional \( \alpha \)-galactoside-binding protein. Furthermore, there is no evidence to suggest that a build-up of \( \alpha \)-galactosides occurs in toxin-treated tissues that renders them susceptible to harmful changes in osmotic pressure (F. Pinkerton, personal communication).

On the contrary, the evidence supports the concept that the toxin-binding protein acts in an indirect manner on the cell. The protein serves in a capacity to recognize the toxin and bind it. Upon binding with the toxin, other functions of the membrane, and subsequently the cell, become altered. For instance, when plasma membranes are exposed to the toxin, there is an increase in ATPase activity (Table 2). This activation of ATPase mediated by toxin-binding protein results in a sudden net increase in K\(^+\) uptake. Further, other membrane enzymes may be activated such as the glycosyl transferase as demonstrated by A. Maretski and M. Thom (personal communication). From this it is conceivable that since at least two enzyme activities are affected by the toxin-binding protein complex, so too is the regulation of the influx and efflux of numerous molecules across the plasma membrane. This is manifested by the fact that water droplets forming on the surfaces of toxin-treated leaves possess a low water potential due to the presence of solutes such as sugars and amino acids (5). A. Novacky (personal communication) has measured transmembrane potential differences with glass microelectrodes in cortical cells of susceptible cane roots. He found an immediate drop in membrane potential upon introduction of the toxin into the experimental system. A similar change was found with giant cells induced by root knot nematode and treated with the toxin. These cells do not possess large vacuoles; hence, the microelectrode tip is in the cytoplasm and the membrane potential measurement is across the plasma membrane per se. This observation would be consistent with the fact that ion efflux from the cell is higher than ion influx and that the ion regulatory function of the plasma membrane is partially or entirely lost.

The low ATPase activities recovered from membrane vesicles in these experiments (Table 2) relative to the comparatively higher activities observed by Leonard and Hodges (7), is attributed to the type of tissues being studied. They used membrane vesicles from oat roots and this current study employed membranes from leaf tissue. Since the root is the site of ion uptake from the soil, it is likely that root tissue could have much more K\(^+\), Mg\(^{++}\) ATPase activity than that of the leaves.

There are several explanations for the toxin-binding protein mediated activation of membrane enzymes. Among these are: (i) a protein-protein complex interaction on the membrane surface wherein one protein binding to its substrate undergoes a conformational change and activates the adjacent protein in the complex, (ii) a direct affect of the toxin on the binding protein which by itself establishes unfavorable ionic and osmotic relationships in the cell. A result of this may be a general perturbation of the entire membrane which renders changes in the activity of membrane enzymes by virtue of a change in their association with the lipid bilayer, and (iii) an indirect affect of the toxin-binding protein complex acting on adjacent membrane enzymes through the lipid phase by means of the induction of a local phase transition in the membrane bilayer, or a phase separation of the bilayer, or conceivably a phase shift in the bilayer. Also, compression-decompression motions of the lipid molecules in the plane of the membrane may explain one membrane enzyme influencing another as described by Singer (16).

Of the three possibilities, any one or more of the statements in point 3 is favored as the explanation for the experimental results. It seems unlikely that assorted activities of a glycosyl transferase, an ATPase, and an \( \alpha \)-galactoside binding protein exists as a membrane complex; this may eliminate point 1. Point 2 is not consistent with current observations, namely that a mild heat treatment protects susceptible leaves from the effects of the toxin (17). Steiner and Byther (17) also showed that new protein synthesis must occur for susceptibility to be restored to the tissues. It turns out that heat treatment abolishes ATPase activity and has no effect whatever on toxin binding activity. These observations collectively support the concept that the production of the toxic effect in tissues is not only dependent upon the binding protein, but also upon an additional protein, perhaps ATPase.

Interestingly, the importance of the aglycone portion of the toxin is realized since the \( \alpha \)-galactosides, raffinose, melibiose, and methyl \( \alpha \)-galactopyranoside do not activate the ATPase through the binding protein (Table 2). Confirmatory data on the ATPase activation effect was apparent in the toxin stimulation of K\(^+\) uptake by cane tissue slices (Fig. 5).

The inherent difficulty in the relative importance of the toxin-stimulated ATPase activation in plasma membrane preparations, is that the effect is observed at a concentration of \( 10^{-4} \) M of toxin whereas in vivo toxin effects on plant leaves occurs in the range of \( 10^{-4} \) to \( 10^{-7} \) M. Furthermore, the toxin
recovered from infected plant tissue is in the order of $10^{-4}$ M (4). The latter figure takes into account the entire liquid content of the tissue. Therefore, it is conceivable that the local external solution of the plant tissue in natural infections could be in the range of $10^{-3}$ M toxin since the toxin does not penetrate the cell. A more likely explanation is that the plasma membrane vesicles in the reaction mixture are not in an optimum physiological state; namely, the normal outer plasma membrane surface may be inside the vesicle, thus barring access to the toxin.

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