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Immunological Comparison of the *in Vitro* and *in Vivo* Labeled Victorin Binding Protein from Susceptible Oats[1]

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ABSTRACT

The fungus *Cochliobolus victoriae* causes victoria blight of oats and produces the host-specific toxin victorin. The reaction of oats to the fungus and its toxin is controlled by a single dominant gene whose product has been hypothesized to function as the site of action (receptor) of the toxin in susceptible oat genotypes. Previously, using a biologically active ^{125}I derivative of the toxin, we identified a 100 kilodalton victorin-binding protein (VBP) which binds victorin in a ligand-specific manner and binds *in vivo* only in susceptible oat genotypes. However, a VBP in both the susceptible and resistant oat genotypes was identified by *in vitro* binding experiments. One interpretation of the lack of genotype-specific binding *in vitro* is that the 100 kilodalton protein detected *in vitro* is not the same 100 kilodalton protein detected *in vivo*. To clarify the relationship between the 100 kilodalton protein(s) labeled *in vivo* and *in vitro*, we developed antisera to the *in vitro*-labeled VBP from the susceptible genotype and demonstrated that these preparations react with the *in vivo*-labeled VBP from the susceptible genotype. This finding coupled with previous observations strongly suggest that the VBP observed *in vivo* is the same protein detected *in vitro*. Furthermore, the results support our previous observations which suggest that the VBPs labeled *in vitro* in susceptible and resistant genotypes are closely related or identical.

The fungus *Cochliobolus victoriae* (anamorph = *Bipolaris victoriae* Shoem.), causal agent of victoria blight of oats (*Avena sativa* L.), produces the host-specific toxin, victorin. Victorin has been characterized as a group of closely related cyclized pentapeptides, the most abundant of which has been designated victorin C (2, 8). Victorin, which is believed to be the primary determinant of pathogenicity, affects only those genotypes of oats carrying the dominant allele at the *Vb* locus. Thus, oat genotypes homozygous for the dominant *Vb* allele are susceptible to the fungus and sensitive to the toxin, whereas homozygous recessive genotypes are resistant to the pathogen and insensitive to the toxin.

It has been hypothesized that the *Vb* gene confers toxin sensitivity and, consequently, pathogen susceptibility by coding for a toxin receptor and that this receptor is either altered or absent in the toxin-insensitive, resistant genotypes (3). Recently, using a ^{125}I-labeled derivative of victorin C, we identified a 100 kD VBP[3], which binds radiolabeled victorin in a ligand-specific manner. Furthermore, the VBP is labeled *in vivo* only in susceptible genotypes. Based on these observations, we have suggested that the VBP is the site of action (receptor) of victorin.

In vitro binding analysis revealed ligand-specific binding to a 100 kD protein from both susceptible and resistant genotypes (5). One possible interpretation of this absence of genotype-specific binding *in vitro* is that the 100 kD VBP observed *in vivo* is not the same protein observed *in vitro*. Thus, the purpose of this study was to determine if the VBP labeled *in vivo* in susceptible genotypes is the same protein labeled *in vitro* in susceptible genotypes. To evaluate this possibility, we compared immunologically the *in vivo*- and *in vitro*-labeled 100 kD VBP from the susceptible genotype.

MATERIALS AND METHODS

Plant Material, Victorin Preparation, and Protein Labeling

Preparation of victorin C and the ^{125}I-BHC, growth conditions for plants, and SDS-PAGE were as previously described (2, 5–8).

In vivo and *in vitro* labeling of oat proteins with ^{125}I-BHC was performed as previously described (5) with the exception that *in vitro* samples, used for indirect immunoprecipitations, were extracted with 400 μL of 80% phenol and precipitated with 2 mL of 0.1 M ammonium acetate in methanol at 4°C overnight. The precipitate was collected by centrifugation, washed with cold methanol, dried under a vacuum, and solubilized in 400 μL of SDS buffer (5). This sample was then used for indirect immunoprecipitations as described below.

Protein samples for Western analysis were prepared from phenol extracts of leaf slices as previously described for *in vivo* binding except that leaf slices were not incubated for 4 h in victorin or ^{125}I-BHC (5).

Protein Purification

Approximately 300 g fresh weight of leaf tissue from the susceptible genotype (X469) was homogenized with a mortar and pestle in 600 mL of buffer containing 0.4 M sucrose, 50 mM Mops (pH 7.5), 2 mM EDTA, and 6 mM 2-mercaptoeth-

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[3] Abbreviations: VBP, victorin binding protein; BHC, Bolton-Hunter derivative of victorin C; WB, wash buffer; TB, transfer buffer; TBST, tris-buffered saline with tween; Pa, 100 kD protein eluted at pH 4.0; Pb, 100 kD protein eluted at pH 5.0.

anol. The homogenate was filtered through four layers of cheesecloth and centrifuged for 1 h at 4°C at 46,000*g* in a Sorvall SS34 rotor (Dupont). The supernatant was discarded and the resulting pellet resuspended in 50 mL of buffer minus sucrose with a Potter-Elvehjem tissue homogenizer. The resulting suspension was centrifuged as above and the supernatant saved. The pellet was resuspended as described above and the process repeated two more times. The resulting supernatants were pooled and centrifuged at 100,000*g* for 1 h. The supernatant was filtered through a Millex-GV 0.22 μm filter (Millipore) and loaded directly onto a Mono Q HR 10/10 column (Pharmacia) equilibrated with 20 mM Tris/HCl (pH 7.5), and 6 mM 2-mercaptoethanol. The column was eluted with a 60-min linear gradient of 0 to 100% 0.5 M Na acetate in equilibration buffer at a flow rate of 1 mL/min. Fractions were collected and analyzed by *in vitro* binding as described earlier (5). Fractions containing the VBP were pooled and stored at −80°C.

The VBP-containing fractions from four individual preparations were combined and dialyzed against 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, and 6 mM 2-mercaptoethanol and then against 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, and 6 mM 2-mercaptoethanol. The dialysate was loaded onto a Mono P HR5/20 column (Pharmacia) and eluted at 1 mL/min for 30 min with Polybuffer 74 (Pharmacia) adjusted to pH 5.0 with HCl. The column was then eluted for 30 min with Polybuffer 74 adjusted to pH 4.0 with HCl. Fractions were collected and aliquots analyzed by SDS-PAGE. Fractions containing a 100 kD protein were found in each buffer elution profile and were well resolved from each other. These fractions were pooled separately. The protein preparation from the more acidic elution conditions (pH 4.0) was referred to as Pa, and the preparation from the less acidic elution conditions (pH 5.0) was referred to as Pb. Pa and Pb were dialyzed against 20 mM Tris/HCl (pH 7.5), 5 mM EDTA, and 6 mM 2-mercaptoethanol, and each was mixed with 0.33 volume of 1.7 M $(NH_4)_2SO_4$, 50 mM sodium phosphate (pH 7.0), and 6 mM 2-mercaptoethanol. Pa was loaded onto a Phenyl Superose HR 5/5 column (Pharmacia) equilibrated in 50 mM sodium phosphate, 0.5 M $(NH_4)_2SO_4$, and 6 mM 2-mercaptoethanol. The column was eluted at 1 mL/min with a 30-min linear gradient of the same buffer without ammonium sulfate. Aliquots of fractions were analyzed by SDS-PAGE, and fractions containing the 100 kD protein were pooled. The Pb sample was subjected to the same chromatographic procedure.

The pooled fractions containing the 100 kD protein from Pa and Pb were concentrated separately in a RCF-ConFilt centrifugal concentrator (Bio-Molecular Dynamics) to a final volume of 0.5 mL and chromatographed on a TSK SWP (7.5 × 75mm) gel permeation column (LKB) equilibrated with 25 mM Tris/acetate (pH 8.3), 5 mM EDTA, and 6 mM 2-mercaptoethanol at a flow rate of 0.3 mL/min. Aliquots of fractions were analyzed by SDS-PAGE, and fractions containing the 100 kD protein were pooled and adjusted to 2.0 mL with running buffer. The total yields were approximately 500 μg of Pa and 330 μg of Pb. Aliquots of 0.5 mL of each preparation were stored at −80°C.

Immunization

A 0.5-mL aliquot of each protein preparation was mixed with 0.6 mL of Freund's complete adjuvant by repeated passage through a narrow gauge syringe needle. Each suspension was then subcutaneously injected in 0.1 mL aliquots at 10 locations into a 7 lb Flemish white rabbit. Two weeks after this initial injection, and at 2-week intervals, each rabbit was given three booster injections prepared and injected the same way except that Freund's incomplete adjuvant was used. Approximately 1 month after the final booster injection, the rabbits were bled by cardiac puncture. The blood was clotted overnight at 4°C, and the serum fraction was clarified by centrifugation. Serum samples were dispensed into aliquots and stored at −80°C until needed.

Control sera were obtained by withdrawing 15 mL of blood from each rabbit before immunization with the antigen. Sera were prepared and stored as described above.

Indirect Immunoprecipitation

Indirect immunoprecipitations were performed with oat leaf protein samples which had been labeled either *in vivo* or *in vitro* with ^{125}I-BHC. The protocol used for indirect immunoprecipitation was basically that of Anderson and Blobel (1). Twenty μL of labeled protein sample was mixed with 20 μL of H_2O. These mixtures were combined with 40 μL of a solution containing 2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl (pH 7.4), and 6 mM EDTA. These solutions were mixed with 400 μL of 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 5 mM EDTA (WB). Five μL of serum were added and the mixture was incubated overnight at 4°C.

The following morning the samples were centrifuged in a microcentrifuge for 3 min and the supernatants transferred to fresh microcap tubes. The samples were mixed with 30 μL of a 50% suspension of protein A agarose (Sigma) which had been presuspended in WB. After 2 h incubation at room temperature with constant shaking, the samples were briefly centrifuged to pellet the protein A agarose, and the supernatant was removed and discarded. The pellets were washed four times with 1 mL of WB and one final time with WB minus Triton and SDS. The final protein A agarose pellets were then suspended in 45 μL of SDS buffer and placed in a boiling water bath for 5 min. The samples were centrifuged and the supernatants analyzed by SDS-PAGE.

Western Blotting

Immediately after SDS-PAGE, gels were equilibrated on a gyratory shaker for 30 min in three consecutive 10-min washes of 100 mL of 25 mM Tris, 192 mM glycine, 0.2% SDS (w/v), and 10% methanol (v/v) (TB). After equilibration, proteins were transferred to nitrocellulose paper at 30 V overnight with an electrophoretic transfer apparatus (Bio-Rad, Trans-Blot) filled with TB. After transfer, the nitrocellulose sheets (blots) were incubated for 1 h in 10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20 (TBST) with 1% BSA. The blots were incubated for 1 h in a 1/500 dilution of serum in TBST with 1% BSA, washed three times for 5 min each in TBST, and incubated for 1 h in a 1/7500 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Promega) in TBST. The blots were washed three times for 5 min each in TBST, blotted between paper towels, and incubated in 100 mM Tris/

HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$ containing a 1/150 dilution of nitro blue tetrazolium at 50 mg/mL in 70% dimethylformamide and a 1/300 dilution of 5-bromo-4-chloro-3 indoyl phosphate (50 mg/mL) in dimethylformamide. After color development, the blots were briefly incubated in 20 mM Tris/HCl (pH 8.0), 5 mM EDTA, and air dried.

RESULTS

Purification of the *in vitro*-labeled VBP resulted in the production of two preparations of protein, Pa and Pb, which appeared homogeneous by SDS-PAGE consisting of a single 100 kD band (Fig. 1). Attempts to monitor *in vitro* binding of ^{125}I-BHC to protein preparations was successful only up to the point of column chromatofocusing (data not shown). After chromatofocusing, preparations failed to bind ^{125}I-BHC. This necessitated the continued purification of the two 100 kD fractions isolated by chromatofocusing. Omission of the chromatofocusing step was not helpful because *in vitro* binding activity was also lost during Superose chromatography.

Indirect immunoprecipitation of *in vitro*- and *in vivo*-labeled VBP (Fig. 2) clearly indicated that antibody raised against both antigen preparations, Pa and Pb, reacted with both the *in vivo*- and *in vitro*-labeled VBP from the susceptible genotype. Furthermore, both antibody preparations reacted with the *in vitro*-labeled VBP from the susceptible and resistant oat genotypes. There was no apparent reaction with control serum from either rabbit.

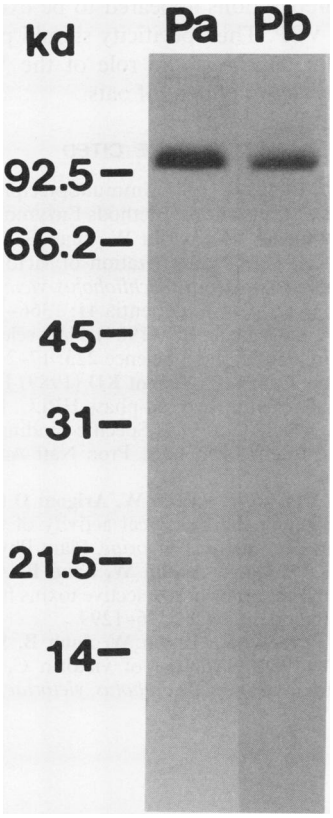

Figure 1. SDS-PAGE of selected fractions from TSK gel permeation chromatography. Pa and Pb indicate the two 100 kD fractions originally resolved by column chromatofocusing. The numbers indicate the migration of molecular mass standards in kD.

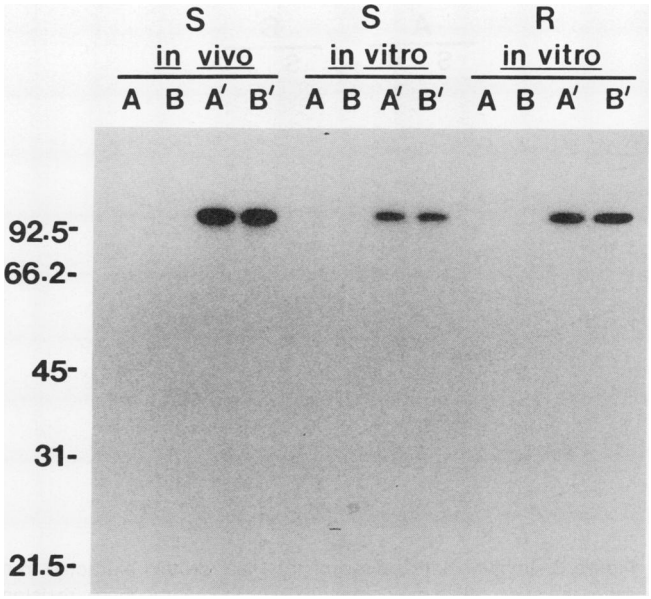

Figure 2. Indirect immunoprecipitation analysis of ^{125}I-BHC-labeled protein from the susceptible oat genotype which had been labeled *in vivo* (S *in vivo*) and *in vitro* (S *in vitro*) and the near-isogenic resistant genotype which had been labeled *in vitro* (R *in vitro*). A and B indicate samples which were incubated with preimmune sera from rabbits. A′ and B′ indicate samples treated with sera from rabbits after immunization with protein preparations Pa and Pb, respectively. The numbers to the left indicate the migration of molecular mass standards in kD.

Western analysis of total protein extracted from oat leaves revealed that antibody raised against both antigen preparations each reacted with a 100 kD protein from both the susceptible and the resistant oat genotypes (Fig. 3). Blots were intentionally overdeveloped to reveal antibody associations with other proteins in the extracts, but no reactions were evident.

DISCUSSION

Previous studies on the binding of ^{125}I-BHC *in vivo* to leaf tissue and *in vitro* to leaf tissue extracts showed that ^{125}I-BHC binds to a 100 kD protein in a ligand-specific manner. *In vivo* binding further demonstrated genotype-specific binding to a 100 kD protein only in the susceptible genotype. However, *in vitro* binding demonstrated ligand-specific binding to a 100 kD protein from both the susceptible and the resistant genotypes (5). The lack of genotype-specific binding *in vitro* suggested the possibility that the protein labeled *in vivo* in susceptible genotypes is not the same as the protein labeled *in vitro* in susceptible genotypes. The results of this study support the conclusion that the VBP labeled *in vivo* is the same protein labeled *in vitro*.

Isolation of the 100 kD *in vitro*-binding protein from the susceptible genotype necessitated the purification of two pro-

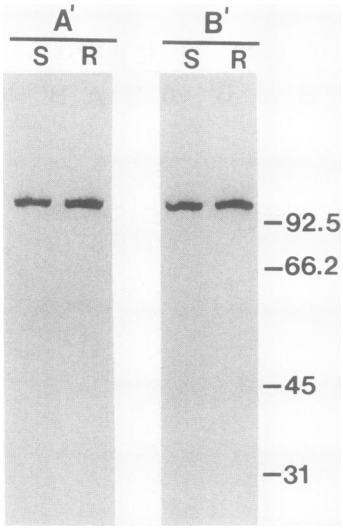

Figure 3. Immunoblot analysis of total leaf protein extracted from either the susceptible oat genotype (S) or the near-isogenic resistant oat genotype (R) and incubated with a 1/500 dilution of antisera raised against protein preparation Pa (A') or preparation Pb (B') followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG and, subsequently, a color-producing substrate. The numbers to the right indicate the migration of molecular mass standards in kD.

tein fractions. The isolation of two protein fractions became necessary because, during the process of purification, protein preparations lost the ability to bind ^{125}I-BHC in a ligand-specific manner. Two 100 kD protein fractions could be resolved on chromatofocusing or Superose columns. The inability to differentiate between these fractions by victorin binding assays following resolution after these chromatographies necessitated the purification of both protein fractions.

The observed loss of victorin binding during the purification process could be due to a variety of factors, *e.g.* protein denaturation, separation from a cofactor which facilitates binding, or protein modifications during purification. However, because antibodies raised against both protein preparations appeared to react identically, the two 100 kD protein preparations probably represent the same protein. The resolution of two fractions during chromatography was possibly due to differential denaturation, a common occurrence in protein chromatography (4). Conditions that resolved the two 100 kD protein fractions also resulted in the loss of ligand-specific binding of ^{125}I-BHC which indicated an association with protein denaturation. However, the possibility of multiple forms of the VBP cannot be dismissed, particularly because *Avena sativa* is a hexaploid and as such could possess multiple alleles for the VBP on other chromosome sets.

Indirect immunoprecipitation analysis clearly demonstrated that antibody preparations directed against both of the 100 kD protein preparations reacted with both the *in vivo*- and the *in vitro*-labeled 100 kD VBP from susceptible genotypes. The observation that these antibody preparations cross-react with the *in vivo*- and *in vitro*-labeled VBP, coupled with the previous observations that both the *in vivo*- and *in vitro*-labeled proteins show ligand-specific binding and have the same molecular mass, strongly suggests that the VBP observed *in vivo* is the same VBP observed *in vitro*.

The specificity of the antibody reaction was demonstrated by Western analysis. Immunoblotting of total leaf protein, resolved by SDS-PAGE, demonstrated that both antibody preparations reacted only with a 100 kD protein from both the susceptible and resistant genotypes.

Although not the stated objective of this study, the indirect immunoprecipitation analysis and the Western immunoblot analysis indicated that antibodies directed against the VBP isolated from the susceptible genotype reacted with the VBP from the resistant genotype. This result is not surprising, because we previously observed that both genotypes bind ^{125}I-BHC *in vitro* in a ligand-specific manner and speculated that the proteins may differ by only a single amino acid residue or, alternatively, may be structurally identical.

Results from immunological comparisons support the conclusion that the VBP observed *in vivo* in susceptible genotypes is the same protein as that observed *in vitro*. Furthermore, the antibody preparations appeared to be extremely specific to the 100 kD VBP. This specificity should provide a useful tool for the elucidation of the role of the VBP in disease development in victoria blight of oats.

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