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High-affinity binding of a protein–lipopolysaccharide phytotoxin from *Verticillium dahliae* to cotton membranes

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The presence of a specific binding site for a protein–lipopolysaccharide (PLP) phytotoxin isolated from culture filtrates of *Verticillium dahliae* has been demonstrated in plasma membranes from cotton seedlings. The ^{125}I-labelled PLP was used as a ligand in binding assays with plasma membrane enriched fractions. Root tissue exhibited the highest amount of binding activity compared to hypocotyl and cotyledon tissue. Binding of the [^{125}I]PLP was saturable, reversible, and with an affinity ($K_d = 1.42 \times 10^{-8}$ M) comparable with the concentration required for biological activity. A single class of binding site was found, and the maximal number of binding sites were estimated as 5.4×10^{-15} mol/μg protein.

Phytotoxin; Protein–lipopolysaccharide; Binding site; Cotton; *Gossypium hirsutum*; *Verticillium dahliae*

1. INTRODUCTION

Virulent strains of the cotton pathogen, *Verticillium dahliae*, produce extra-cellular high molecular weight protein–lipopolysaccharide (PLP) complexes that are phytotoxic and can induce most of the symptoms associated with *Verticillium* wilt disease [1,2]. The recognition mechanisms that signal the presence of potential pathogens and the transmission of extra-cellular signals in plant cells are still poorly understood. The key event in the induction of defense responses in plants is the interaction of the signal molecule with the presumed primary target site(s). Various sites, including the cell wall, the plasmalemma, and some intra-cellular components have been suggested as putative targets for the primary action of elicitors [3]. Previous reports have identified binding proteins for fungal phytotoxins and fungal elicitors on plant cell membranes [4–7]. Here we report on the identification of a saturable and high-affinity binding site in cotton plasma membrane enriched fractions for a protein–lipopolysaccharide phytotoxin isolated from culture filtrates of a virulent strain of *Verticillium dahliae*.

2. MATERIALS AND METHODS

2.1. *Plant material*

The cotton (*Gossypium hirsutum* L.) cultivar OR19 was used in the study. This cultivar exhibits resistance towards *Verticillium dahliae*, the causative agent of *Verticillium* wilt in cotton.

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2.2. *Plasma membranes*

Plasma membranes were isolated from cotton seedlings grown for 7 days in the dark at 25°C in vermiculite. Seedlings were carefully removed, washed, and placed on ice. The tissue (roots, hypocotyls and cotyledons) was cut into small sections of approximately 0.5 cm in length. The plant tissue preparation (10 g) was then placed into 25 ml of ice-cold homogenization media which contained 250 mM sucrose, 3 mM ethylenediaminetetraacetic acid (EDTA), 2 mM adenosine 5′-triphosphate (Na_2ATP), 10% (v/v) glycerol, 1.0% (w/v) bovine serum albumin (fraction V), 0.5% (w/v) polyvinylpyrrolidone (PVP, M_r 40,000) 2 mM phenylmethylsulphonyl fluoride (PMSF), 15 mM β-mercaptoethanol, 4 mM dithioerythreitol (DTE), 250 mM KI, and 70 mM Tris-HCl, pH 8.0. DTE, PMSF, and β-mercaptoethanol were added to the medium just prior to use. The homogenization medium was then vacuum infiltrated into the tissue for 5 min at ice temperature prior to homogenization as described by Giannini et al. [8]. The homogenate produced by this method was filtered through six layers of cheesecloth and then centrifuged at 13,000 × *g* for 3 min in a microcentrifuge at 4°C. The pellet was discarded and the supernatant was centrifuged at 13,000 × *g* for 25 min in the microcentrifuge to obtain a microsomal membrane pellet [8]. When used directly in binding assays, the microsomal membrane pellets were suspended in 0.33 M sucrose, 5 mM Tris-MES, pH 6.5.

2.3. *Sucrose density gradient centrifugation*

The microsomal pellets were suspended in 500 μl suspension buffer, (250 mM sucrose, 10% glycerol, 1 mM DTE and 1 mM PMSF (added fresh), and 2 mM bis-Tris propane/MES, pH 7) and 400 μl was layered on a discontinuous sucrose density gradient consisting of 700 μl of 25% (w/v) sucrose layered over 300 μl of 35% (w/v) sucrose (both sucrose solutions contained 1 mM Tris/MES, pH 7.2, and 1 mM DTE) in a 1.5 ml microcentrifuge tube. The gradients were centrifuged for 1 h at 13,000 × *g* and the plasma membranes at the 25%/38% interface [8] were removed and stored at −70°C for up to 2 weeks or used immediately. H^+ATPase activity associated with the plasma membrane was monitored to determine purity of the fractions [8], which varied between 82% and 86%.

2.4. *Purification of the PLP complex*

The macromolecular PLP complex from *Verticillium dahliae* was prepared from seven-day-old culture filtrates (Meyer and Dubery,

unpublished). The purification procedure consisted of 80% acetone precipitation and gel permeation chromatography on Sepharose CL-6B, (2.5 × 60 cm column) using distilled water as eluant. The phytotoxic fractions was concentrated and the PLP was further purified by preparative agarose (1.5%) gel electrophoresis in 20 mM NH_4HCO_3, pH 8.0. The eluted PLP (M_r 194,000) was electrophoretically homogeneous on a 5% polyacrylamide gel (Fig. 1); and was found to be stable for up to four weeks at 4°C. The PLP complex consisted of 16% protein, 14% lipid and 70% carbohydrate. Further details on the purification and characterization are to be published elsewhere.

2.5 Radio-labelling of the PLP complex

The electrophoretic homogeneous PLP was radiolabelled with ^{125}I according to the method of David [9], using immobilized lactoperoxidase coupled to CNBr-activated Sepharose to a final concentration of 2.3 mg enzyme per ml of settled beads. Any remaining reactive groups on the beads were exposed to 0.2 M glycine, 0.01 M phosphate, pH 7.5 at 4°C for 5 h. After thorough washing the Sepharose-bound enzyme was stored in phosphate-buffered saline, pH 7.4 containing 10^{-5} M merthiolate.

Iodinations were carried out at room temperature as follows: the Sepharose-bound enzyme (30–40 μl) was washed twice with 1 ml of 0.01 M Tris-HCl buffer through centrifugation (2,000 × g). The PLP solution (250 μl) containing 1 mg/ml protein, was pipetted into the tube after the final wash. Two μl of 2 mM KI was added, followed by 2 μl $Na^{125}I$ (3.7 MBq/μl). The iodination reaction was initiated by the addition of 15 μl 2 mM H_2O_2. Further 5 μl aliquots were added in 5 min intervals up to 30 min. The Sepharose-bound enzyme was kept suspended by manual agitation throughout the procedure. The reaction was terminated by the addition of 10 μl of 2.5 M NaN_3. The Sepharose beads were removed by centrifugation. The supernatant containing the [^{125}I]PLP and free ^{125}I ions was subjected to desalting on a Sephadex G-25 gel filtration column eluted with water. 10 μl aliquots of 1 ml fractions were counted on a LKB Compugamma gammacounter. The specific radioactivity of the product was 954.6 GBq/mmol.

2.6. Binding assays

The assays to determine binding of ^{125}I-labelled PLP to plasma membranes were performed in binding assay buffer (20 mM Tris-MES, pH 7.0, containing 0.25 M sucrose and 5 mM $MgSO_4$) as follows: the plasma membranes were suspended in binding assay buffer at 25°C. Unless otherwise indicated, incubation mixtures contained plasma membranes (10 μg of protein) and ^{125}I-labelled PLP (2.5–100 nM) in a total volume of 200 μl of binding assay buffer in 1.5 ml microcentrifuge tubes. Samples were incubated in a water bath for 2 h at 25°C. Unbound labelled PLP was removed on a Millipore Millititer system with cellulose-acetate membranes. Each sample was washed with 10 volumes of binding buffer by rapidly filtering the solution under vacuum. The filters were punched out and the amount of radioactivity remaining on the filters was measured directly using a LKB 1282 Compugamma counter. Non-specific binding of [^{125}I]PLP to membranes was assayed by performing the above procedure with a 800-fold excess of non-labelled PLP. All assays were performed in triplicate. Specific binding was calculated as the difference between total and non-specific binding.

3. RESULTS AND DISCUSSION

The presence of PLP binding sites in total membrane fractions isolated from different parts of the seedlings (root, hypocotyl and cotyledon) of the OR19 cotton cultivar was detected by incubating increasing amounts of each membrane preparation with [^{125}I]PLP. The data in Fig. 2 show that all three membrane preparations had some ability to bind the radiolabelled toxin. Membranes prepared from cotton roots exhibited the highest amount of PLP binding activity per μg of membrane protein. The binding activity present in the hypocotyl and cotyledon tissues was approximately half of that present in the root tissue. Further characterization of the binding site was therefore carried out with enriched plasma membrane vesicles from root tissue, purified by sucrose density gradients.

The pH dependence of radiolabelled PLP binding to membranes was investigated and optimum binding was observed from pH 6.5 to 8 (data not shown). Binding of toxin to the membranes dropped off rapidly at pH values less than 6 and greater than 8. Based on these results, binding assays were routinely performed in 20 mM Tris-HCl, pH 7.0. The optimum temperature for binding was determined as 30°C.

The saturability of the binding of [^{125}I]PLP to the plasma membrane binding sites was examined by incu-

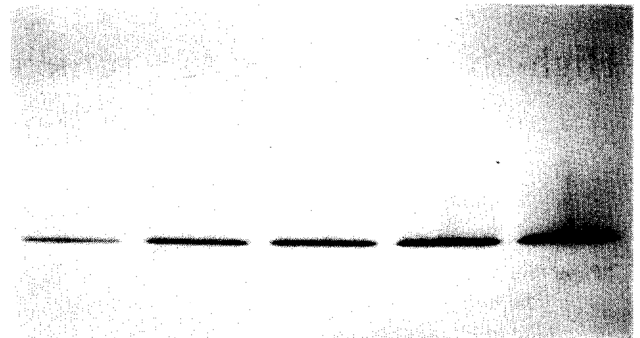

Fig. 1. 5% Polyacrylamide gel electrophoresis of the purified protein–lipopolysaccharide phytotoxin from *V. dahliae*. The lanes correspond to increasing amounts (2.5–10 μg) of the purified 194 kDa phytotoxin.

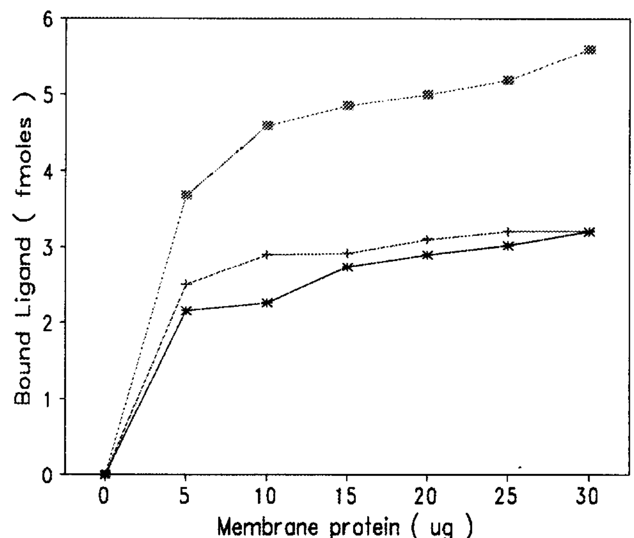

Fig. 2. Binding of [^{125}I]PLP to plasma membrane fractions prepared from various cotton tissues. Increasing amounts of membranes prepared from root (■), hypocotyl (+) and cotyledon (×), were incubated with 10 nM [^{125}I]PLP in a total volume of 200 μl. The binding assays were performed as described in section 2.

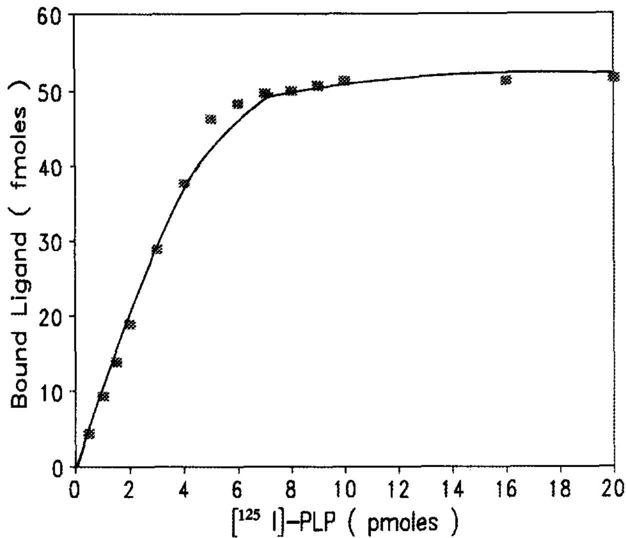

Fig. 3. Binding isotherm of [^{125}I]PLP to plasma membranes from cotton roots. Increasing amounts (2.5–100 nM) of radiolabelled PLP were added to membranes (10 μg) and incubated as described in section 2. Non-specific binding was determined in the presence of a 800-fold excess of unlabelled PLP. Each point represents the average of three replicates.

bating root membranes with increasing concentrations of the radiolabelled PLP. The result of these experiments, shown in Fig. 3, demonstrate that the binding was saturable. Analysis of the specific binding by the methods of Scatchard [10] (Fig. 4), indicated the presence of a single class of binding site with an apparent K_d of 1.42×10^{-8} M which is indicative of high affinity binding sites. This value correlated well with the minimum amount PLP needed (1.29×10^{-8} M) to induce a hypersensitive reponse, characterized by wilting and necrosis, in the OR19 cultivar of cotton (Meyer and Dubery, unpublished). The maximal number of binding sites, apparent site concentration or B_{max}, was 5.4×10^{-15} mol/μg protein. The Hill plot [11] (not shown) of the specific binding data was linear, indicating the presence of a single class of PLP binding site; and the Hill coefficient of 0.72 suggests that there is some negative cooperativity associated with binding.

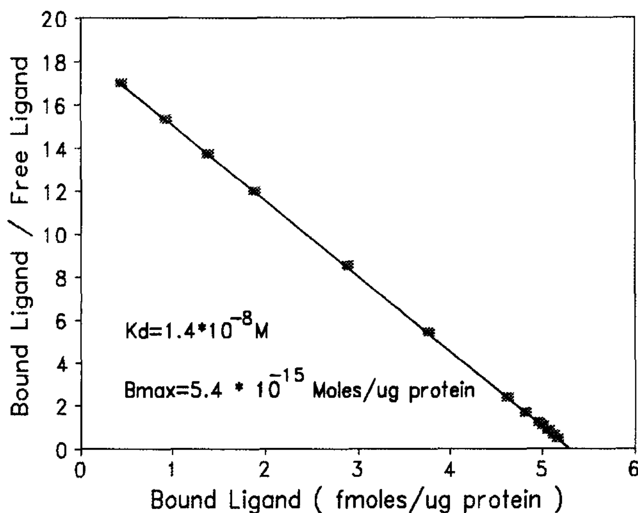

Fig. 4. Scatchard analysis of the specific binding of [^{125}I]PLP to cotton root membranes. The specific binding data shown in Fig. 3 was used to calculate the plots shown. The apparent dissociation constant (K_d) and maximal number of binding sites B_{max}, were calculated by least-squares analysis.

The binding characteristics determined in this study is comparable to that obtained with the interaction between a glucan elicitor from *Phytophthora megasperma* and soybean membranes (K_d = 37 nM, B_{max} = 1.2 pmol/mg protein) [12], and that for the binding between a hepta β-glucoside elicitor and a putative receptor from soybean membranes (K_d = 75 nM, B_{max} = 1.2 pmol/mg protein) [13].

Fig. 5 shows the kinetics of binding and dissociation of the [^{125}I]PLP with the plasma membrane localized binding site. Association of the labelled PLP to its binding site was more rapid than dissociation, half-maximum binding was achieved at 30°C within 42 min after adding the ligand. In contrast, dissociation of the radiolabelled PLP from the binding site, in the presence of excess (1,000-fold) unlabelled ligand, was half complete after 75 min.

This report is the first demonstration of the occurrence of high affinity, specific binding sites for the protein lipopolysaccharide fungal toxin from *Verticillium dahliae* in plasma membranes from tissues of the host plant. It contributes to a better understanding of the molecular mechanism of the interaction between cotton and *Verticillium*. We are currently extending these investigations to evaluate the importance of the PLP bind-

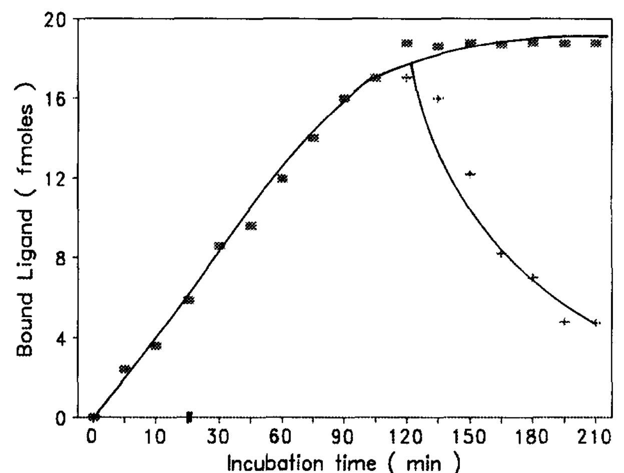

Fig. 5. Kinetics of binding and dissociation of [^{125}I]PLP to plasma membrane fractions from cotton roots. Association kinetics (■) were determined by adding labelled PLP (10 nM) at time 0 to root membranes (10 μg of protein) and incubating the samples for increasing amounts of time. Dissociation kinetics (+) were determined by adding a 1000-fold excess of unlabelled PLP 2 h after starting the incubation with labelled PLP and incubating the samples further for the time period indicated. Non-specific binding was measured by adding a 1000-fold excess of unlabelled PLP simultaneously with the labelled ligand at time 0. Each point represents the average of three replicates.

ing sites in cotton membranes against the background of resistance and susceptibility and pathogen recognition.

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