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Peptide mimotopes of phomopsins: Identification, characterization and application in an immunoassay

Meng Yu*, Khin Than, Steve Colegate, Brian Shiell, Wojtek P. Michalski, Stephen Prowse & Lin-Fa Wang
CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia
*(*Author for correspondence, E-mail: meng.yu@csiro.au, Tel: +61-3-52275027, Fax: +61-3-52275555)*

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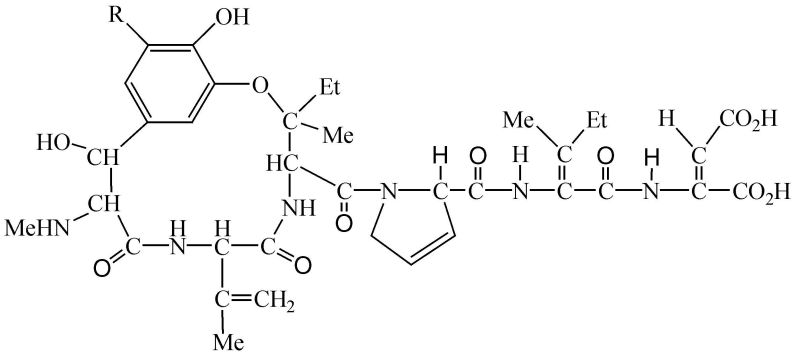
Summary

Peptide mimotopes of plant-associated toxins offer the potential for improving analytical and diagnostic methodologies as well as providing candidates for potential protective vaccines against plant poisoning diseases. Monoclonal antibody (mAb) C_3C_{11}, which recognizes the antimicrotubule phomopsin mycotoxins, was used to isolate peptide mimics of phomopsin A from a random 15-mer phage display peptide library. A total of 46 clones were isolated that showed specific reactivity with the mAb. Amino acid sequence analysis revealed four different types of mimotope sequences, all of which contained a common motif V-A-L/V-C. Of the 46 clones isolated, 44 contained the motif V-A-L-C while 2 contained the V-A-V-C motif. All four types of phage clones inhibited the reactivity of the mAb with phomopsin A in a competition ELISA. The clone with the mimotope sequence CT**VALC**NMYFGAKLD demonstrated the strongest binding. It was further shown that synthetic peptides containing these mimotope amino acid sequences were able to inhibit the mAb-phomopsin A interaction, indicating that the peptide mimotopes were responsible for the specific binding, independent of the phage framework. The results also suggest that the mimotope peptides bind to mAb C_3C_{11} at the same site as phomopsin A. The application of recombinant phage particles carrying phomopsin mimotopes in immunoassay was evaluated and the results demonstrated approximately 100-fold increase in sensitivity in comparison with a conventional immunoassay using a chemically linked phomopsin-horseradish peroxidase conjugate.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, immunoglobulin; M13, filamentous bacteriophage M13; mAb, monoclonal antibody; MW, molecular weight; PBS, phosphate buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine

Introduction

Poisoning by low molecular weight natural toxicants causes considerable economic loss in livestock production worldwide [1]. Increasing attention is also being paid to human health risks posed by the possible transfer of natural toxicants into animal products consumed by humans [2, 3]. The hepatotoxic phomopsins comprise a family of hexapeptides, containing a 13-membered ring formed by an ether bridge, produced by the fungus *Diaporthe toxica* (formerly *Phomopsis leptostromiformis*), and are the causative agents of lupinosis in domestic livestock [4–6]. Phomopsin A ($C_{36}H_{45}ClN_6O_{12}$, see structure 1 in Figure 1), the main mycotoxin, is a linear hexapeptide containing 3-hydroxy-L-isoleucine, 3,4-didehydro-L-valine, *N*-methyl-3-(3-chloro-4,5-dihydroxyphenyl) serine, *E*-2, 3-didehydro-aspartic acid, *E*-2, 3-didehydroisoleucine, and 3,4-didehydro-L-proline [7–9]. Phomopsin A induces mitotic abnormalities in the liver with associated histopathology. Early detection of contaminated pasture and lupin seeds is an important measure to reduce the incidence of lupinosis. In comparison with other analytical methods developed for phomopsin detection, immunoassays have several advantages for rapid field-testing, including high specificity and sensitivity, facile sample preparation, and ease of use. Enzyme-linked immunosorbent assay (ELISA) using phomopsin conjugated to horseradish peroxidase (HRP) is currently the method of choice for detection of phomopsins. However, the efficiency of the chemical conjugation process is low. Utilisation of a specific replacement for the



[1] R = Cl phomopsin A

[2] R = H phomopsin B

Figure 1. Structure of phomopsin molecules. Depending on the composition of the R group, phomopsins can be divided into two types, A and B.

phomopsin-HRP conjugate, such as a peptide mimic of the phomopsins, has the potential to improve the ELISA assay.

Since the publication of three seminal papers describing the first generation of phage display random peptide libraries in 1990 [10–12], this technology has attracted a great deal of interest as a powerful tool to study protein–protein interaction and to isolate peptide mimics for a variety of biological molecules. Phage display peptide libraries are constructed by inserting random peptide coding sequences in front of the *N*-terminus of phage coat proteins, so that the "in-frame-fused" peptides are displayed on the phage surface upon expression and assembly. A typical random peptide library contains millions or even billions of different peptide sequences, each displayed by a separate phage clone. Phage display peptide libraries have been used to identify ligands for peptide receptors [13] and virus capsid [14], to define epitopes for monoclonal antibodies [12], to select enzyme substrates [15], and to select peptide mimics of non-proteinaceous molecules such as biotin [16] and carbohydrates [17], among a myriad of other applications [10, 18–21].

It is of interest to determine whether peptide mimics of low-molecular-weight mycotoxins could be identified using phage peptide display technology. This report describes the characterization of four closely related 15-mer peptide mimotopes of phomopsin A, which were selected from a phage-displayed random peptide library, and the comparison of these mimotopes with the conventional phomopsin conjugate in an ELISA format for the sensitive and specific detection of phomopsin in experimental and field samples.

Materials and methods

Reagents

All inorganic chemicals and organic solvents were reagent grade. Bovine serum albumin (BSA) was purchased from Trace Biosciences (New Zealand), phosphate buffered saline (PBS) from Oxide (England), polyoxyethylene sorbitan monolaurate (Tween 20), fetuin, tetramethylbenzidine (TMB) and horseradish peroxidase (HRP) from Sigma (USA), Centricon-10 microconcentrator and YM 10 and YM 100 membranes from Amicon (USA), dimethyl sulfoxide from Merck Pty. Ltd (Germany) and methanol from BDH Laboratory Supplies (England). Cell culture reagents were purchased from ICN Biomedicals (USA). Goat anti-mouse IgG- HRP conjugate was purchased from Silenus Labs Pty. Ltd (Melbourne). Mouse anti-M13-HRP conjugate, Streptavidin-HRP and Protein G Sepharose were purchased from Amersham Biosciences (Sydney, NSW, Australia).

Production of monoclonal antibody against phomopsin

The immunogenic conjugate was prepared via a water-soluble carbodiimide method. Thus, phomopsins (9 mg, 11 μmol comprising 80–85% Phomopsin A) were dissolved in water (5 mL, pH approx. 8) by slowly introducing a small volume ammonia vapor. Fetuin protein (15 mg, 0.31 μmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (40 mg, 209 μmol) were added and the solution stirred for 24 h at room temperature. Unconjugated phomopsins were removed by ultrafiltration using a YM10 membrane filter (10,000 MW cutoff). The molar ratio of attachment in the retentate, based on unbound phomopsins in the filtrate (absorbance at 278 nm), was estimated to be 12 phomopsins:1 fetuin. The retentate was redissolved in water (30 mL) and stored as 0.5 ml aliquots (equivalent to 50 μg phomopsins and 250 μg fetuin) at -20°C.

Four female Balb/c mice, six to eight weeks old, were immunised with the phomopsins-fetuin protein conjugate. Each animal was injected subcutaneously with the conjugate (0.1 mL, equivalent to10 μg phomopsins and 50 μg fetuin) emulsified in an equal volume of Freund's complete adjuvant and divided equally between two injection sites. The mice received three additional injections of the same amount of conjugate emulsified in Freund's incomplete adjuvant at 21 days

intervals. Blood from the tail vein was taken prior to the first immunisation and 10 days following the fourth immunisation. The production of antibodies against phomopsins was assessed using an indirect ELIA [22]. Briefly, 100 μL of phomopsin (0.5 μg/mL in carbonate buffer, pH 9.6) was used to coat 96-well microtitre plate (Nunc^{TM}, Denmark), and mouse sera were tested with serial dilution in assay buffer (0.1% gelative in PBS) starting from 1:100. Bound antibodies were detected anti-mouse IgG -HRP and TMB substrate. Four days prior to the preparation of the hybridomas, two mice showing strong antibodies against phomopsin were injected intraperitoneally with the same amount of conjugate but without adjuvant.

Spleen cells from each mouse were harvested and fused with murine myeloma NS-1 cells using 50% polyethylene glycol. Hybridomas were selected by hypoxanthine, aminopterin and thymidine and grown as per Harlow and Lane [23]. Hybridomas were assessed for anti-phomopsin antibody production on day 12. Hybridomas secreting antibodies to phomopsins were selected using a competitive ELISA (see more detail below). Positive cultures, thus identified were cloned by limited dilution, on an average of 2, 1, 0.5 and 0.25 cells/well. Further assessment of phomopsin antibody production in the clones was done only on wells with a single cell colony in each well. Positive cells were grown, recloned and frozen in culture media containing 10% DMSO and stored in liquid nitrogen. Monoclonal antibodies were prepared from ascitic fluid from 6 to 8 weeks old female Balb/c mice [23]. The antibodies were purified using Protein G Sepharose 4 Fast Flow column (Amersham Biosciences, Stockholm) and concentrated by ultrafiltration using Amicon YM100 membrane, both following protocols supplied by the respective companies. The anti-phomopsin IgG concentration in PBS pH 7.3 was determined by its absorbance at 280 nm (0.75 mg purified IgG/mL = 1 optical density).

Competitive ELISA for anti-phomopsin monoclonal antibody determination

PolySorp 96-well microtitre plate (Nunc^{TM}, Denmark) was coated with 100 μL of phomopsin at a concentration of 0.5 μg/mL in carbonate buffer pH 9.6, and incubated overnight at 37 °C. After four washings with washing buffer (PBS containing 0.05% Tween), 50 μL of undiluted culture supernatant was added into the wells with 50 μL in assay buffer (0.1% gelatin in PBS) containing 1 ng of phomopsin or with 50 μL of assay buffer alone. After incubation at room temperature for 2 h, the plate was washed four-times with washing solution, and the bound monoclonal antibodies were detected using anti-mouse IgG -HRP and TMB substrate. The level of phomopsin-specific antibodies was determined by the ratio of the optical density in wells with and without free phomopsin (Optical density $B/B_0 \times 100$).

Chemical conjugation of phomopsins to horseradish peroxidase

Phomopsins-HRP conjugate was also prepared *via* the water-soluble carbodiimide method at pH 8.5 as follows: HRP (10 mg, 0.25 μmol) in water (0.75 mL) adjusted to pH 8.5 by addition of 0.05 M NaOH was mixed with a solution of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide-HCl (16.5 mg, 86 μmol) in water (0.75 mL) at pH 8.5. A crystalline mixture of phomopsins (0.79 mg, 1 μmol) purified from cultures of *Diaporthe toxica* on lupin seed [5] was then added. After stirring for 24 h at room temperature, free phomopsins were removed by ultrafiltration using a Centricon-10 microconcentrator (10,000 MW cutoff). The retentate was washed four times with 2 mL H_2O at pH 8.5. The degree of conjugation was estimated (phomopsins: HRP, 1.4:1) from absorbance measurements at two wavelengths, 400 nm for HRP and 278 nm for the phomopsins, respectively. The retentate was redissolved in 3 mL water and further diluted at 1:20 in 40% glycerol in PBS pH 7.3 with 0.1% thimerosal and stored in 1 mL aliquots at −18 °C. Further dilution was made as necessary before use.

Selection of mimotopes from phage display peptide library

The fUSE5-15-mer (linear, non-constrained) phage display peptide library was kindly provided by Dr G. P. Smith, University of Missouri, Columbia, USA, together with bacterial strains and protocols. The library contained 1.3×10^9 individual members. Panning was conducted essentially as given by Smith and Scott [24]. Thus, for coating, 100 μL of anti-phomopsin monoclonal antibody (mAb C_3C_{11}) at a concentration of 68 μg/mL in Tris-NaCl buffer (0.05 M Tris-HCl, 0.15 M of NaCl, pH 9.0) was dispensed into each well of a MaxiSorp 96-well microtitre plate (Nunc^{TM}, Denmark). Blank wells were coated with the same amount of a control mAb 20D11, against the VP7 protein of bluetongue virus [25]. The plate was incubated for 1 h at 37 °C with shaking and stored at 4 °C overnight. The wells were washed three-times with PBST buffer (0.01 M phosphate-buffered saline with 0.05% Tween 20, pH 7.4). Non-specific binding was blocked by adding 200 μL of Blotto solution (2% skim milk in PBST) to each well and the plate was incubated at 37 °C for 1 h, followed by three washes with PBST. Approximately 2.3×10^{10} phage particles, diluted in 100 μL Blotto solution, were added into each well and the plate was incubated at room temperature for 2 h with shaking. The plate was then washed five-times with PBST and five-times with PBS (0.01 M phosphate-buffered saline, pH 7.4), respectively. To elute the bound phage, 100 μL of phomopsin A solution (5 μg/mL in PBS containing 1% methanol) was added to each well and the plate was incubated at room temperature for 30 min with shaking at low speed. The eluate was removed from each well and used to infect *E. coli* strain K91/Kan for phage titration and amplification as previously described by Smith and Scott

[24]. Three rounds of panning were conducted using same conditions.

Phage ELISA

The wells of a 96-well PVC microtiter plate were coated with 50 μL of either mAb C_3C_{11} or control mAb 20D11 [25] at a concentration of 3.4 μg/mL using the same coating buffer and incubation conditions as described above. The wells were washed three-times with PBST between each of the following incubation steps, which were carried out at 37 °C for 1 h with shaking: (i) the wells were firstly blocked with 200 μL of Blotto, followed by (ii) incubation with 50 μL of phage solution from individual clones and (iii) detection with 50 μL of the anti-M13-HRP conjugate diluted at 1:3000 in PBST. Finally, color development was carried out by incubation with 50 μL of TMB at room temperature for 10 min. The absorbance at 450 nm was determined after the reaction was stopped by adding 50 μL/well of 1 M H_2SO_4.

DNA sequencing of selected phage inserts

PCR was used to amplify the insert region of the phage genome encoding the peptide sequence with phage DNA released from picked colonies by boiling. The following vector-specific primers were used for PCR: upstream primer (FUSE-U) GCA AGC TGA TAA ACC GAT AC and downstream (FUSE-D) CCA TGT ACC GTA ACA CTG AG. The amplified fragments were sequenced by the Big-Dye (Applied Biosystem, Foster City, USA) dideoxy termination cycle sequencing using the upstream primer and analyzed on an ABI 377 automatic sequencer. Sequence management and analysis were done using the Clone Manager suite of programs (S&E Software, Durham, USA)

Synthetic peptides

Synthetic peptides SP3-8 (Biotin-KGWGDVALCDPLLPK-OH), SP3-8s (Biotin-KGWGDVALSDPLLPK-OH), and SP1-6 (Biotin-GKKGCTVALCNMYFGAKLD-OH) were commercially made by Mimotopes (Clayton, Australia). A biotin tag was added to the *N*-terminus of each peptide to facilitate peptide detection in ELISA. For peptide SP1-6, four additional charged residues, GKKG, were added at the N-terminus to increase the solubility of the original peptide. Peptides were dissolved in water at 1 mg/mL and stored at −20 °C as stock solution.

Competition ELISAs

Competition ELISAs (C-ELISAs), in the presence of phomopsin A at different concentrations, were conducted to determine the binding of the coated mAb C_3C_{11} with either (i) phage mimotope, (ii) phomopsin-HRP conjugate or (iii) biotin-peptide. In all three cases, the coating, washing, incubation and color development conditions were the same as those described previously for the phage ELISA. The remaining steps, specific for each of the C-ELISAs were as follows: (1) Phage mimotope C-ELISA: equal volume of phage solution (diluted 1:10,000 in TBS) and phomopsin A at different concentrations (diluted at 0 to1250 ng/mL in PBS-1% methanol) were mixed before use and 50 μL of the mixture was added to each well. The bound phage was detected by incubation with 50 μL/well of the anti-M13-HRP conjugate (diluted 1:3000 in PBST). (2) Phomopsin-HRP C-ELISA: similar to (1) with two modifications, i.e., replacing the phage solution with the phomopsin-HRP conjugate at 1:50 in PBS-1% methanol, and remove the addition of anti-M13-HRP. (3) Synthetic peptide C-ELISA: similar to (1) with two changes. The phage solution was replaced with synthetic peptide at 50 μg/mL in ddH_2O, and the anti-M13-HRP was replaced with streptavidin-HRP (diluted at 1:1000 in PBST).

Preparation of lupin seed extracts

Phomopsins-contaminated lupin seed (50 g) or clean lupin seed (50 g) were soaked in 250 mL of methanol:water (4:1) in a 500 mL glass bottle. After steeping overnight at room temperature, 100 mL of the extraction solution were transferred to a clean container. The seed and the remaining 150 mL of extraction solution were transferred into a stainless steel chamber of Omni-Mixer Homogenizer (USA). To prevent excessive heating, the chamber was kept in ice during the 3 min homogenizing at high speed. Macerated seed suspension was transferred back into the original bottle. The chamber was washed with the previously saved 100 mL of extraction solution and combined with the macerated seed. After stirring for 2 h, the extraction solution was centrifuged at 2000 g for 15 min and the supernatant was stored at −18 °C until ready to be assayed. The extract was then diluted to a minimum of 1:10 in PBS-1% methanol assay buffer during the assay protocol.

Results

Isolation and characterization of phomopsin mimotopes

In order to isolate peptides that can mimic the phomopsin mycotoxins, the anti-phomopsin monoclonal antibody (mAb C_3C_{11}) was used to pan a phage-displayed peptide library containing 15-aa peptide inserts fused to the phage minor coat protein pIII of up to five copies per virion. After each round of panning, enrichment of specific phage clones binding to the selecting mAb was observed by calculation of phage recovery ratio. After the third round of panning, 96 individual clones were randomly picked and propagated for phage production. Phage preparations from 46 clones showed specific reactivity with mAb C_3C_{11} in the phage ELISA, having a reading at least two-fold higher than that obtained with negative control mAb 20D11 (data not shown). The amino acid sequences for

Table 1. Comparison of mimotope sequences deduced from ELISA positive clones

Mimotope type	Sequence	Representative clone	No. of clones (%)
1	CT**VALC**NMYFGAKLD	PM1-6	25/46 (54.3%)
2	GWCD**VALC**DPLLP	PM2-7	16/46 (34.8%)
3	GWGD**VALC**DPLLP	PM3-8	3/46 (6.5%)
4	PSYCPAPAVSI**VAVC**	PM4-9	2/46 (4.3%)

these 46 positive clones are summarized in Table 1. Four different types of insert sequences were observed, all of them containing the motif V-A-L/V-C. Three types of inserts, representing 44 out of the 46 clones, include the motif V-A-L-C and the other two clones from the 4th type have the motif V-A-V-C. It is interesting to note that among the mimotope clones containing the V-A-L-C motif, 41 out of 44 clones also contained a cysteine (C) residue upstream, resulting in the motif C-x-V-A-L-C (x = T in type 1, and D in type 2). Further analysis using synthetic peptides indicated that these C residues were apparently important to maintain the structural features to mimic phomopsin in binding to mAb C_3C_{11} (see below).

Comparison of phomopsin and phage mimotopes in their binding to mAb C_3C_{11}

Binding studies were carried out using competition ELISA (C-ELISA) to determine whether the binding by positive mimotope-containing phage clones was due to a true mimicking of the phomopsin epitope recognized by mAb C_3C_{11} or due to non-specific binding to the antibody regions outside of the antigen-binding site (the paratope). Four phage clones, representing each of the four different types of mimotope sequences, were chosen for this study, and these clones were designated PM1-6, PM2-7, PM3-8, and PM4-9, respectively (see Table 1). The antigen concentration of each phage preparation was determined using the phage ELISA and the concentration that gave rise to an absorbance reading of approximately 1.5 was chosen for C-ELISA analysis. Similar optimization was also carried out for the phomopsin-HRP conjugate. Specific inhibition of mAb-phage or mAb-phomopsin-HRP binding was determined in the presence of different concentrations of free phomopsin A. The results presented in Figure 2 indicated that (i) as for phomopsins-HRP, the binding of each of the four phage mimotopes could be specifically inhibited by free phomopsin A; (ii) there was no significant difference among the inhibition curves for the four different phage mimotope clones; (iii) there was a clear difference between the inhibition curves of phage mimotopes and that of the phomopsins-HRP (Phom-HRP), with the phage inhibition curves showing a steeper slope; and (iv) phage mimotope binding was inhibited by lower concentrations of phomopsin A than was the binding of phomopsin-HRP. There was also a small, but significant difference in the maximal inhibition achieved. Depending on the inhibition level, about 100-fold less phomopsin A was required to achieve the same level of inhibition for phage than that for phomopsins-HRP, e.g. approximately 0.125–0.25 ng free phomopsin per well resulted in 70–80% inhibition of phage mimotope binding, whereas 8–31.5 ng per well was required for that of phomopsins-HRP binding.

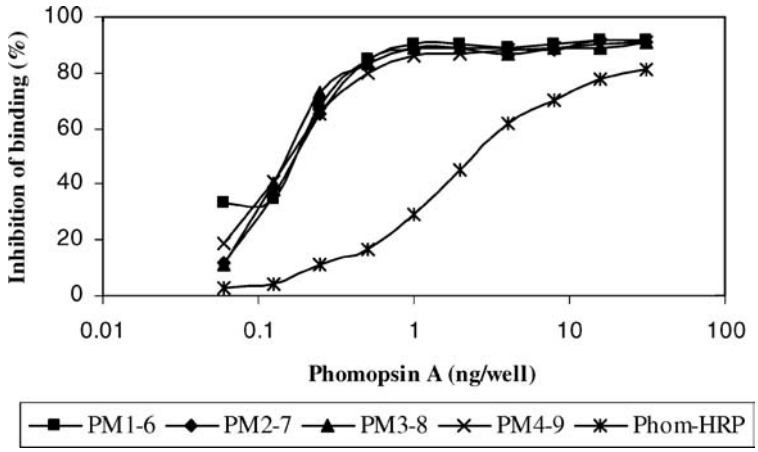


Figure 2. Competition between phage mimotopes and free phomopsin A for binding to immobilized mAb C_3C_{11}. The binding of representative mimotope clones (see Table 1) to mAb C_3C_{11} was studied in the presence of different concentrations of free phomopsin A. A titration was first conducted for each of the phage preparation to determine the phage dilution which gave rise to an OD reading of approximately 1.0 in the phage ELISA. Percentage inhibition of mimotope binding was plotted versus the concentration of phomopsin A. Phomopsin-HRP conjugate (phom-HRP) was used as a control.

Table 2. Sequences of synthetic peptides

Peptide name	Sequence (original mimotope sequence given in bracket)	Original phage clone
SP1-6	Biotin-GKKGCT**VALC**NMYFGAKLD–OH (CT**VALC**NMYFGAKLD)	PM1-6
SP3-8	Biotin-KGWGD**VALC**DPLLP (GWGD**VALC**DPLLP)	PM3-8
SP3-8s	Biotin-KGWGD**VAL**SDPLLP (GWGD**VALC**DPLLP)	PM3-8

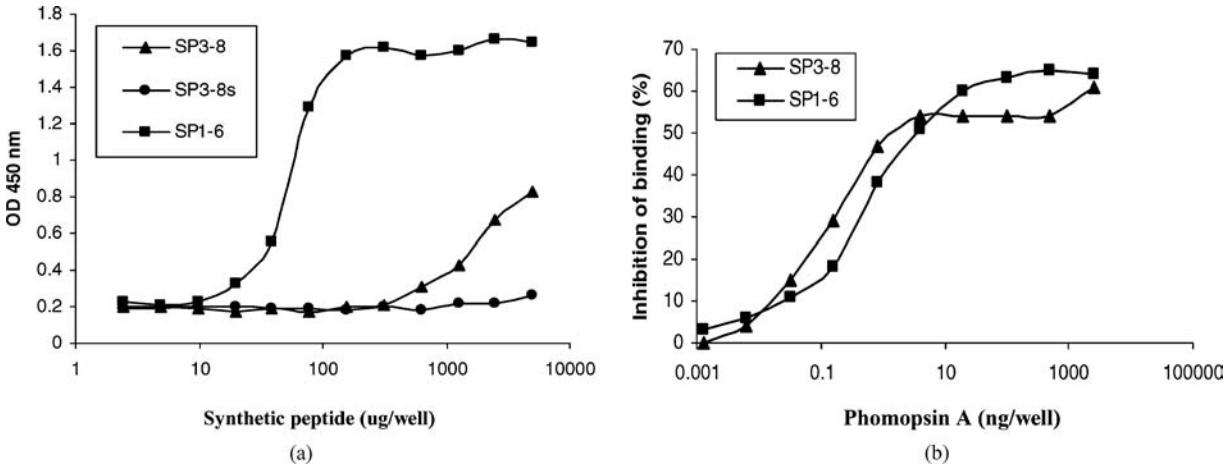


Figure 3. Binding of synthetic peptides to immobilized mAb C_3C_{11}. (a) Binding of biotinylated peptide mimotopes to the immobilized mAb was monitored by HRP-conjugated streptavidin. (b) Inhibition of peptide-mAb binding in the presence of different concentrations of phomopsin A. To obtain an OD of approximately 1.0, it was necessary to use more SP3-8 (at 100 μg/mL) than SP1-6 (at 1 μg/mL).

Binding characteristics of synthetic peptides

To confirm that the peptide sequences truly mimicked the structural features of phomopsin and thus bind specifically to mAb C_3C_{11}, independent of the phage structural context, the binding characteristics of three synthetic peptides (see Table 2 for peptide sequences) were investigated. Examination of their mAb-binding properties by ELISA demonstrated that peptides SP3-8 and SP1-6, both containing the intact V-A-L-C motif, were able to bind to the mAb albeit at different levels, whereas peptide SP3-8s, in which the cysteine residue was replaced by a serine, showed no binding (Figure 3a). This suggests that the C residue in the V-A-L-C motif is essential for binding to the mAb. It is also clear from Figure 3a that peptide SP1-6 had much higher reactivity than SP3-8. To further confirm that the synthetic peptide-mAb interaction is a true mimic of phomopsin-mAb binding, C-ELISA was conducted in the presence of different concentrations of phomopsin A. The results are shown in Figure 3b. Despite the fact that peptides SP1-6 and SP3-8 showed significant difference in their reactivity with mAb C_3C_{11} (Figure 3a), their inhibition patterns by phomopsin were very similar. These results confirmed the previous C-ELISA analysis using phage clones and demonstrated that the synthetic peptides were able to maintain the mAb-binding activity independent of the phage framework.

Incorporation of the phage-displayed mimotope into a C-ELISA for detection of phomopsin in lupin seed extract

The results shown above in Figures 2 and 3 suggested that the use of mimotope-displaying phage seemed to be more sensitive than the conventional phomopsin-HRP in detecting free phomopsin, and that it is therefore feasible to replace the phomopsin-HRP conjugate with a mimotope-displaying phage or a biotinylated mimotope peptide for the development of a C-ELISA. Using clone PM1-6 and the same C-ELISA conditions previously described in this report, different samples of lupin seed extracts were tested. These included both "clean" and "*Diaporthe toxica*-contaminated" lupin seeds as well as the free phomopsin A as positive control. As shown in Figure 4, a concentration-dependent inhibition was observed for both the phomopsin A control and the "contaminated" seed sample, but not for the "clean" seeds.

Discussion

This study has demonstrated that peptide mimics of low molecular weight toxin molecules can be isolated from phage display random peptide libraries. The strategy of using free phomopsin molecules for elution resulted in a highly effective

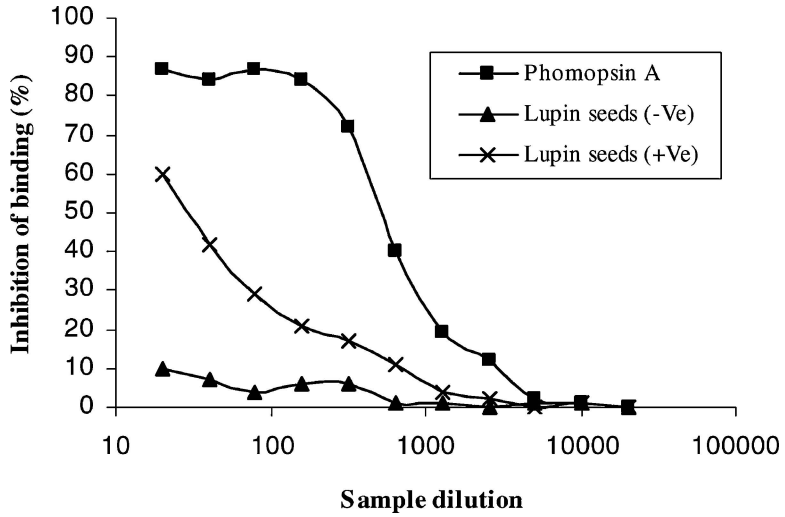


Figure 4. Detection of phomopsin from field samples using a phage mimotope-based C-ELISA. The level of phomopsin contamination in seed samples was monitored by their ability to inhibit the binding of the mimotope-displaying phage to immobilized mAb C_3C_{11}. Free phomopsin A was included as a control.

selection of phomosin mimotopes. After three rounds of panning, more than 50% of clones were confirmed to contain functional mimotopes of the toxin molecules.

Out of the forty six clones sequenced, four different types of phomopsin mimotopes from a phage display random peptide library were identified each carrying the common motif V-A-L/V-C. In competition ELISAs, free phomopsin A could compete with the phage-displayed mimotopes for binding to mAb C_3C_{11}, suggesting that these mimotopes bind to the same antigen-binding site of the mAb. Furthermore, specific, phomopsin-dependent binding of synthetic peptides containing these mimotope sequences, but free of the phage context, to the mAb was also demonstrated. These results indicated that the mimotope peptides alone, without involvement of the phage coat framework, were sufficient for their binding to the mAb. For the V-A-L/V-C motif, we have shown that the change of the Cys residue to Ser in peptide SP3-8s abolished the binding of mimotope PM3-8 (Figure 3a). We have also observed that the mimotope PM1-6, showing the best binding to the mAb, has an additional upstream Cys residue in the hexapeptide sequence **C**TVAL C. It is interesting to note that the formation of an intramolecular disulfide bond between the two Cys residues would form a ring structure, highly similar to that of the phomopsin molecule (Figure 1). Analysis of the peptide SP1-6 solution (in water) by mass spectrometry indicated that approximately 15% of the peptides were in the oxidized disulfide-bonded form (data not shown). However, more study is required to determine whether the oxidized form of the peptide performs better than the reduced form in the C-ELISA.

It has been reported that the structures of mimotope peptides of non-proteinaceous molecules, especially those of carbohydrates, tend to include aromatic side chains and proline residues [17, 26]. This was also true for mimotopes of the mycotoxin deoxynivalenol [27]. However, the mimotope peptides identified in this study did not show such preference.

When phage clone PM1-6 was used directly in a C-ELISA replacing the phomopsin-HRP conjugate, it was shown that the C-ELISA performed well not only for free phomopsin A, but also for field samples of contaminated seed extracts. Although mimotope peptide-HRP conjugate has been used in immunoassays [27, 28], to our knowledge this is the first demonstration of a phage-displayed mimotope directly applied to a toxin detection assay. This novel application has several advantages over the conventional chemical conjugation method: (i) as shown in this study, the assay is more sensitive than the use of toxin-HRP conjugate due to the amplification effect provided by the multiple copies of the major phage coat proteins linked to the mimotope peptides; (ii) the "biological conjugation" of mimotope to phage coat proteins is uniform and reproducible. Although the titers of different phage preparations may vary from batch to batch, there was no variation in C-ELISA performance once the phage antigen concentration is normalized (data not shown); (iii) recombinant phage are easy to produce and very cost effective (a 10-mL culture produced enough phage for 10,000 assays in this study); (iv) use of a non-toxic phage solution also makes the assay safer from the occupation health point of view. It is evident that our approach is equally applicable for any low molecular weight compounds that are hard to obtain or unsafe to use in ELISA.

Finally, it should be noted that mimotope peptides are also excellent candidates for vaccine development. Previous studies have provided proof of principle for prevention of toxicity by immunization using toxin conjugated to different carrier proteins [29]. The identification of toxin mimotopes provides new opportunities in vaccine development, such as the use of DNA vaccination and other novel strategies that have been tried for carbohydrate vaccine development [30, 31].

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