Synthesis of Protein Conjugates and Development of Immunoassays for AAL Toxins

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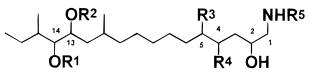
AAL toxins and fumonisins, produced by *Alternaria alternata* f. sp. *lycopersici* and *Fusarium moniliforme*, respectively, are sphinganine-analogue mycotoxins with important health implications. Highly sensitive, inexpensive, and rapid immunoassays are needed to quantify these natural toxins in food products and animal feeds. We developed novel methods for the synthesis of protein conjugates of the AAL toxin TA. Mice were immunized with the conjugates, and the resulting polyclonal antisera were used to devise class-selective ELISAs for AAL compounds. The most selective assays had detection limits for the AAL toxins in the low parts per billion range with no significant cross-reactivities for a number of structurally similar compounds, including fumonisin B_1 and sphinganine. Our selected conjugates are candidate immunogens to produce monoclonal antibodies and further polyclonal antisera for the detection of the AAL toxins.

Keywords: *AAL toxins; fumonisins; synthesis of conjugates of AAL toxin TA; polyclonal mouse antisera; enzyme immunoassay; ELISA; cross-reactivity*

INTRODUCTION

AAL phytotoxins, produced by the fungal pathogen *Alternaria alternata* f. sp. *lycopersici*, are tricarballylic esters of a series of long-chain aminopolyols structurally related to sphinganine (Bottini et al., 1981; Caldas et al., 1994, 1995; Boyle et al., 1994; Oikawa et al., 1994; Boyle and Kishi, 1995) (Figure 1). Each of the five major toxin fractions (TA, TB, TC, TD, and TE) consists of a pair of regioisomer esters (Figure 1).

Fumonisins (e.g., FB_1, Figure 2), mycotoxins structurally related to AAL toxins, originally were isolated from maize colonized by *Fusarium moniliforme*, a common pathogen of maize and other grains (Nelson et al., 1993). Consumption of fumonisin-contaminated maize has been reported to induce maladies ranging from cancer to renal, neural, and hepatic necrosis in several animal species and may be responsible for human esophageal cancer (Thiel et al., 1992; Riley et al., 1994; Norred and Voss, 1994). FB_1 and AAL-toxin TA induced genotype-specific cell death in tomato lines isogenic for the *Asc* gene (Gilchrist et al., 1992), and both compounds were found to be cytotoxic to cultured mammalian cells (Mirocha et al., 1992). Recent evidence indicates that cell death in both plant and animal systems triggered by both TA and FB_1 toxins shows stereotypical hallmarks of apoptosis (Wang et al., 1996a,b). While the signal transduction pathways leading to either cell death or cell proliferation induced by these toxins are unknown, current studies suggest that interference with early steps in sphingolipid biosynthesis (Riley et al., 1994; Abbas et al., 1994; Wu et al., 1995) may play an important role in initiating the cellular responses observed. Both AAL toxins and fumonisins are specific inhibitors of the sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase) enzyme in animals (Merrill et al., 1993; Wang et al., 1996a) and plants (Abbas et al., 1994).



Toxin	R^1	R^2	R^3	R^4	R^5
TA_1	H	R	OH	OH	H
TA_2	R	H	OH	OH	H
TB_1	H	R	H	OH	H
TB_2	R	H	H	OH	H
TC_1	H	R	H	H	H
TC_2	R	H	H	H	H
TD_1	H	R	H	OH	$COCH_3$
TD_2	R	H	H	OH	$COCH_3$
TE_1	H	R	H	H	$COCH_3$
TE_2	R	H	H	H	$COCH_3$

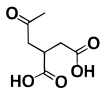


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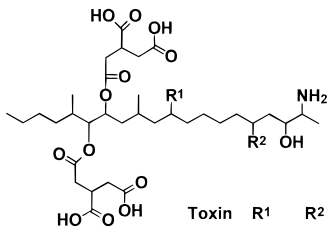
Figure 1. Structures of AAL toxins. Each major toxin fraction is composed of a pair of regioisomers, e.g., TA is a mixture of TA_1 and TA_2.



Toxin	R^1	R^2
FB_1	OH	OH
FB_2	H	OH
FB_3	OH	H

Figure 2. Structures of fumonisins FB_{1-3}.

The structural and toxicological similarity of the AAL toxins and fumonisins and the presence of both fungal pathogens in harvested plant products have raised concern about the contamination of human foods and

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animal feeds by these mycotoxins (Caldas et al., 1994). Concentrations of these sphinganine-analogue toxins in plant matrices usually range between low parts per billion and low parts per million values (Thiel et al., 1992; Usleber et al., 1994; D. G. Gilchrist, unpublished), which are sufficient for representing a potential hazard to human and animal health.

Highly sensitive, selective, inexpensive, and rapid analytical methods for both classes of mycotoxins are required for further biological studies and routine screening of foodstuffs and animal feeds. Quantitative detection of *Alternaria* toxins currently is limited to instrumental methods such as high-performance liquid chromatography (HPLC) (Siler and Gilchrist, 1982) and mass spectrometry (MS) (Caldas et al., 1995). Similar techniques including capillary zone electrophoresis (CZE) have been used to determine fumonisins (Pestka et al., 1994; Ware et al., 1994; Caldas et al., 1995; Maragos, 1995). Most of these analyses share some drawbacks: they usually require costly apparatus, extraction, cleanup, and derivatization procedures; their sample throughput is limited; and they are not suitable for on-site analysis. In addition, most HPLC, TLC, and CZE detection techniques are based on derivatization of free amino groups which precludes detection of the acetylated TD and TE AAL toxins. Several enzyme-linked immunosorbent assays (ELISAs) are now available for measuring fumonisins at ppb−ppm levels (Azcona-Olivera et al., 1992; Fukuda et al., 1994; Usleber et al., 1994; Shelby et al., 1994; Chu et al., 1995; Elissalde et al., 1995). These immunoassays are class-selective for fumonisins and adaptable for monitoring large numbers of samples. Preliminary results for the development of ELISAs for AAL toxins have been reported previously (Szurdoki et al., 1996).

MATERIALS AND METHODS

Safety Note. Fumonisins and AAL toxins are potential carcinogens. Both groups of toxins should be handled with care.

Reagents. Common reagents and organic solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Immunochemicals, proteins, and analytical standards of high quality were obtained from Sigma Chemicals Co. (St. Louis, MO) unless otherwise stated. The sodium salt of sulfosuccinimidyl 4-(4-(*N*-maleimido)phenyl)butyrate (sulfo-SMPB) was purchased from Pierce (Rockford, IL). Succinimidyl 6-(*N*-maleimido)hexanoate (MHS) was supplied by Boehringer Mannheim Co., Biochemical Products (Indianapolis, IN). The AAL toxins were obtained and their structure and purity (≥95%) was confirmed as reported previously (Bottini et al., 1981; Caldas et al., 1994, 1995). Pure aminopolyol (HTA, $R_1 = R_2 = R_5 = H$ and $R_3 = R_4 = OH$ in the structure in Figure 1) was produced by the hydrolysis of TA in 2 N KOH at 70 °C for 2 h according to Mirocha et al. (1992). Aqueous solutions were prepared with water (16.7 MΩ/cm) obtained from Sybron/Barnstead Nanopure II system (Barnstead Co., Newton, MA).

Instruments. Electrospray mass spectra (ES-MS) were recorded by a VG Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, U.K.), as described by Caldas et al. (1995). ELISA experiments were performed in 96-well microplates (NUNC, Roskilde, Denmark; Model 442404) and using a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). Softmax (Molecular Devices) software was employed for fitting the sigmoidal standard curves based on Rodbard's (1981) four-parameter logistic method and for interpolation of unknown sample concentrations.

Syntheses of Protein Conjugates. In the five sets of protein derivatives **N1**−**N5** (Figures 3−6), TA toxin was conjugated to native proteins through its amino group. In the synthesis of conjugates **C** (Figure 7), the carboxyl group(s) of compound TA was activated and linked to proteins. These

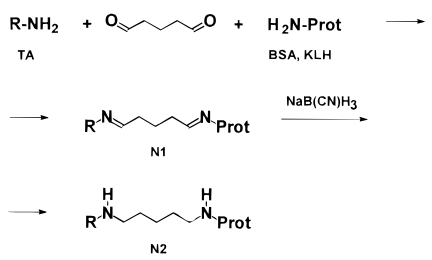

Figure 3. Synthesis of **N1** and **N2** conjugates.

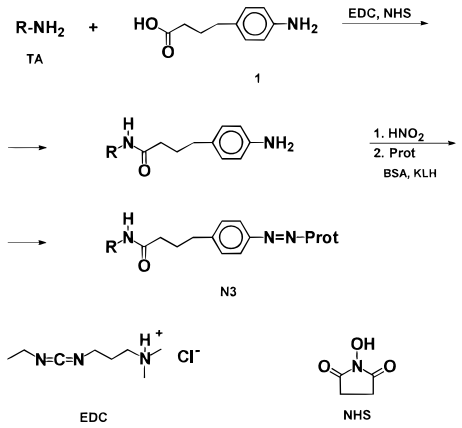

Figure 4. Synthesis of **N3** conjugates.

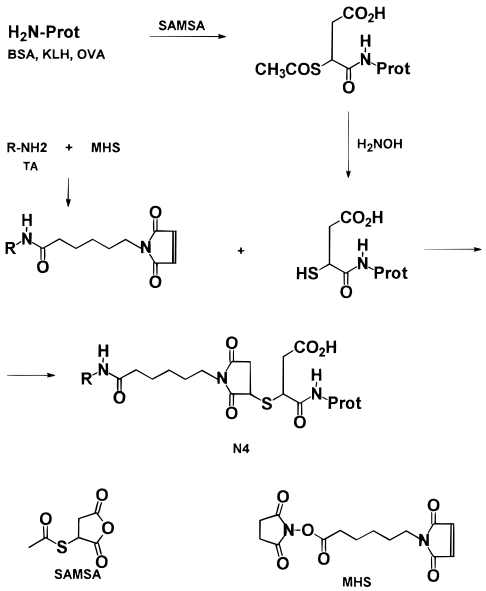

Figure 5. Synthesis of **N4** conjugates.

experiments were carried out in glass vials with Teflon lined screw caps with magnetic stirring. Dialyses were performed against about 4.5 L of buffer at 4 °C unless otherwise stated. Exhaustive dialysis involved five changes of buffer.

***N1** Conjugates (Glutaraldehyde Coupling without Reductive Stabilization).* Bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) were derivatized to produce **N1**−BSA and **N1**−KLH conjugates, respectively (Figure 3). The carrier protein (10 mg), dissolved in 12 mL of water with stirring, was activated by dialyzing it against 2 L of 0.2% glutaraldehyde in 0.01 M phosphate buffer plus 0.8% NaCl, pH 7.5 (phosphate-buffered saline, PBS) for 20 h. Excess reagent was removed by dialyzing the same bag three times against PBS, after which the contents were transferred into a glass vial. TA (5 mg, 9.6 μmol), dissolved in methanol (0.30 mL), was added dropwise to the stirred activated protein solution at 4 °C, and stirring was continued at the same temperature for 20 h. Aqueous L-lysine hydrochloride solution (0.2 M, 0.1 mL) was then added dropwise to block unreacted aldehyde groups. The reaction mixture was stirred at room

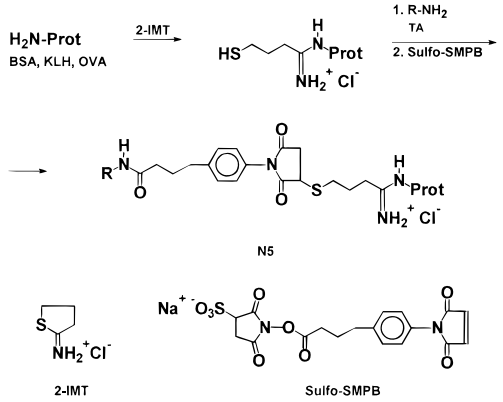

Figure 6. Synthesis of **N5** conjugates.

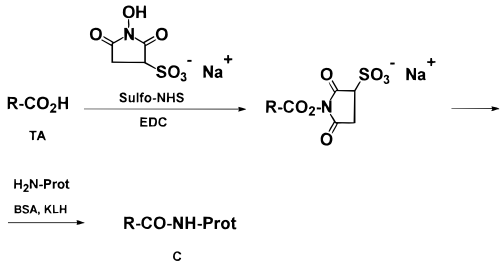

Figure 7. Synthesis of **C** conjugates.

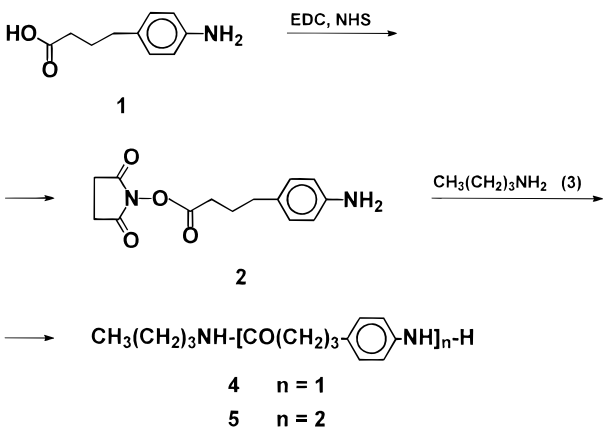

Figure 8. Model reaction forming 1-butylamide (**4**) of 4-(4-aminophenyl)butyric acid (**1**).

temperature for an additional 4 h. One-third of the reaction product was purified by exhaustive dialysis in PBS to yield **N1** protein conjugate. The remaining two-thirds of the reaction mixture was used directly in the preparation of the corresponding **N2** conjugate.

N2 *Conjugates (Products Obtained by Reductive Treatment of the Glutaraldehyde Conjugates).* **N1**−BSA and **N1**−KLH conjugates were used to furnish the **N2**−BSA and **N2**−KLH conjugates, respectively (Figure 3). Sodium cyanoborohydride (95%, 16.5 mg, 0.25 mmol) was added to the two-thirds part of the **N1**-reaction mixture with stirring that was continued at ambient temperature for 20 h. The resulting conjugate was purified by exhaustive dialysis in PBS.

N3 *Conjugates (Attachment of TA Toxin to Proteins by Cross-Linker **1**).* 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (98%, 4.3 mg, 22 μmol) was added to 4-(4-aminophenyl)butyric acid (**1**, Figures 4 and 8; Freedman and Doak, 1949; 3.6 mg, 20 μmol), *N*-hydroxysuccinimide (NHS) (97%, 2.9 mg, 24 μmol), and dry tetrahydrofuran (THF; 1.5 mL) with stirring under nitrogen at 4 °C. The reaction mixture was stirred rapidly under nitrogen at the same temperature for 2 h and then at room temperature overnight. A small aliquot of the reaction mixture was added to excess ice-cooled 1-butylamine (25 mM solution in 0.13 M bicarbonate buffer, pH 8), stirred at room temperature overnight, extracted with ether, dried over sodium sulfate, and evaporated in vacuo. The residue was taken up in acetonitrile, and analyzed by ES-MS in positive mode (ES^+-MS). The sample was applied by direct inlet using acetonitrile as solvent. ES^+-MS (*m*/*e*): 235 ($M + H^+$, 100%), 396 ($M + C_{10}H_{11}NO + H^+$, 5%). The parent peak at 235 mass unit ($C_{14}H_{22}N_2O + H^+$) indicated that the expected 1-butylamide (**4**) of compound **1** was the main component of the sample. The peak at 235 mass unit ($C_{24}H_{33}N_3O_2 + H^+$) corresponds to the 1-butylamide (**5**) of the dimer of compound **1**, which was a minor impurity.

Meanwhile, a solution of TA (8.4 mg, 16 μmol) in methanol (0.5 mL) and then the bulk of the above reaction mixture containing the NHS ester of acid **1** (**2**, Figure 8) in THF was added dropwise to a rapidly stirred 0.13 M sodium bicarbonate solution (8 mL) at 4 °C. The reaction mixture was stirred at the same temperature for 1 h and then at room temperature overnight. A temperature of 4 °C was maintained unless otherwise stated during the following operations. The reaction mixture was first neutralized by adding 5 N hydrochloric acid dropwise with stirring, the mixture was stored overnight, and then the solution was adjusted to pH 1 with 0.5 M sulfuric acid. Sodium nitrite (2.1 mg, 30 μmol) was added to the reaction mixture. After stirring for 30 min, urea (3.6 mg, 60 μmol) was added, then after 5 min the reaction mixture was divided into two equal portions. These aliquots were added immediately to the cooled and rapidly stirred protein solutions (12.5 mg each of BSA and KLH in 20 mL of 0.15 M $Na_2B_4O_7 \cdot 10H_2O$ [borax] solution). The mixtures were stirred at 4 °C overnight and then at room temperature for 2 h before exhaustive dialysis in PBS to yield the yellow **N3**−BSA and **N3**−KLH conjugates.

N4 *Conjugates (Attachment of TA Toxin to Proteins by Cross-Linker MHS).* In this synthesis, the amino group of TA was acylated by a cross-coupling reagent, succinimidyl 6-(*N*-maleimido)hexanoate (MHS). The resulting TA−MHS derivative was then linked to thiolated proteins.

The solution of *S*-acetylmercaptosuccinic anhydride (SAMSA, 96%, 45.4 mg, 0.25 mmol) in dimethylformamide (DMF, 1 mL) was added dropwise to the rapidly stirred solution of carrier protein (150 mg) in 0.2 M phosphate buffer, pH 8 (30 mL) at 4 °C. The reaction mixture was stirred at room temperature for 1 h; the acetylmercaptosuccinylated protein conjugate was then exhaustively dialyzed in PBS.

MHS (10 mg, 32.5 μmol) in methanol (5 mL) and 0.1 M phosphate buffer, pH 8, (5 mL) were added to a solution of TA (14.3 mg, 27.4 μmol) in methanol (0.85 mL) at 4 °C with stirring. The reaction mixture was stirred at 4 °C overnight, diluted with water (5 mL), stirred for 2 h at 4 °C, and then washed with 2 × 5 mL of 2:1 hexane/ethyl acetate mixture to remove excess MHS. The aqueous reaction mixture, containing TA derivatized by MHS, was divided into three equal parts; then nitrogen was bubbled through the aliquots for 15 min.

The acetylmercaptosuccinylated protein conjugate solution (24 mg in 6 mL of PBS) and a solution of 0.33 M hydroxylamine hydrochloride plus 20 mM ethylenediaminetetraacetic acid (EDTA) in 0.2 M phosphate buffer, pH 7.5 (3 mL), were purged with nitrogen for 15 min. The two solutions were then combined and stirred under nitrogen at room temperature for 30 min to liberate thiol groups. An aliquot of the solution of TA−MHS derivative prepared above was added dropwise to this solution containing the thiolated protein without delay. The reaction mixture was stirred under nitrogen in a sealed vial overnight and then dialyzed exhaustively in PBS. BSA, KLH, and ovalbumin (OVA) were derivatized in this way; the products were designated as **N4**−BSA, **N4**−KLH, and **N4**−OVA, respectively.

N5 *Conjugates (Attachment of TA Toxin to Proteins by Cross-Linker sulfo-SMPB).* In this conjugation reaction, TA was incubated with the thiolated protein and the cross-linker, sulfo-SMPB (Kamps-Holtzapple et al., 1993, 1994). Nitrogen was bubbled through the solution of the carrier protein (15 mg) in PBS plus 0.1 M EDTA, pH 8 (PBS-EDTA) (4 mL) for 15 min. 2-Iminothiolane hydrochloride (2-IMT, Traut's reagent) (99%, 4.2 mg, 30 μmol) was added to this solution and the reaction mixture stirred under nitrogen in a sealed vial at room temperature for 1 day. Aqueous glycine solution (1 M, 0.4 mL)

was added to destroy excess reagent with stirring. The reaction mixture was dialyzed against PBS−EDTA. The resulting solution of the thiolated protein was combined with the TA solution (3.0 mg, 5.8 μmol) in methanol (0.3 mL); nitrogen was bubbled through the solution briefly followed by the addition of the sodium salt of sulfo-SMPB (3.0 mg, 6.5 μmol). The reaction mixture was stirred under nitrogen in a sealed vial at room temperature overnight and then dialyzed exhaustively in PBS. BSA, KLH, and OVA were conjugated using this method; the products were designated as **N5**−BSA, **N5**−KLH, and **N5**−OVA, respectively.

C *Conjugates (Attachment of TA Toxin to Proteins by N-Hydroxysulfosuccinimide plus EDC).* Water (2 mL) and the monosodium salt of (±)-1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonic acid (*N*-hydroxysulfosuccinimide or sulfo-NHS) (97%, 4.2 mg, 18.8 μmol) was added to the ice-cooled, rapidly stirred solution of TA (3.9 mg, 7.5 μmol) in 0.2 mL of methanol and adjusted to pH 4 with diluted aqueous hydrochloric acid at 4 °C. EDC (98%, 3.7 mg, 18.9 μmol) was added to this solution and the reaction mixture was stirred at 4 °C for 20 min, while pH 4 was maintained by addition of diluted hydrochloric acid or sodium hydroxide solutions. The mixture was then immediately added dropwise to a stirred solution of 10 mg of carrier protein in 10 mL of 0.13 M sodium bicarbonate solution at 4 °C. The reaction mixture was stirred at the same temperature for 2 h and at ambient temperature overnight followed by exhaustive dialysis in PBS. BSA and KLH were treated by this method; the conjugates were designated as **C**−BSA and **C**−KLH, respectively.

Production of Mouse Polyclonal Anti-TA Antibodies. Male Swiss Webster mice (18−20 g) were purchased from Simonsen Laboratories (Gilroy, CA). After 1 week of acclimation, a preimmune-bleed sample (0.1 mL) was taken and then three mice were injected intraperitoneally with each immunogen protein conjugate. The immunogen, dissolved in PBS, was emulsified with Freund's complete adjuvant (1:1 volume ratio). Each mouse was injected with 50 μg of protein conjugate in 0.1 mL volume. After 30 days, the mice were boosted with an additional 50 μg of immunogen in PBS mixed with Freund's incomplete adjuvant (1:1 volume ratio). Ten days following the first boost, a test bleed (0.1 mL) was taken followed by a second boost by the same method as the first boost. Ten days later another test bleed was taken. This schedule was followed until titers appeared to reach a maximum. The blood samples were centrifuged at 800*g* in an Eppendorf microcentrifuge and the antisera removed and kept frozen at −20 °C until used.

ELISA Experiments. Enzyme immunoassay studies were done following standard procedures for indirect ELISAs in the coating antigen format. Homologous and heterologous assay systems were screened in direct binding studies. The concentration of selective antibody (titer) was considered high if the absorbance values exceeded 2 under conditions similar to those used in the procedure below except that the inhibitor was omitted. Assays with absorbance values of less than 0.3 were not used for further work. Selected antiserum-coating antigen combinations with adequate titers were optimized by two-dimensional titrations and studied in competition experiments as follows.

Each well of a 96-well microplate was coated with 100 μL of the appropriate coating antigen concentration (usually 1 μg/mL) in 0.1 M carbonate−bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The coated plate was washed with PBSTA (phosphate-buffered saline plus 0.05% Tween 20 plus 0.02% sodium azide, pH 7.5). Different concentrations of inhibitors, obtained by serial dilutions of an appropriate stock solution with PBS, 50 μL of each, were dispensed to the wells. An appropriate dilution (usually 1/1500) of the anti-AAL antiserum in PBSTA was prepared, and 50 μL of this solution was also added to each well. The plate was incubated at room temperature for 2 h and then washed thoroughly. Goat antimouse IgG conjugated to alkaline phosphatase (Sigma), diluted in PBSTA buffer (1/2500), was added (100 μL/well), and the plate was incubated at room temperature for 2 h. After washing the plate, 100 μL/well of *p*-nitrophenyl phosphate (Sigma, 1 mg/mL) in substrate buffer (10% diethanolamine plus 0.1 mg/mL $MgCl_2 \cdot 6H_2O$, pH 9.8) was added. The plate was incubated for 30 min at room temperature; then the absorbance at 405 nm minus absorbance at 650 nm was determined. The dose−response curves were analyzed to calculate IC_{50} values, i.e., analyte concentrations (ppb) required for 50% inhibition.

RESULTS AND DISCUSSION

In preliminary experiments, we attempted to adapt a fumonisin-conjugation method from the literature (Azcona-Olivera et al., 1992) for the development of ELISAs for AAL compounds, but only antibodies of poor affinity were obtained (data not shown). Thus, the AAL toxins appeared to be difficult targets and a multiconjugate−multiantibody approach seemed advisable in order to develop useful immunoassays for AAL toxins. Polyclonal mouse antisera were raised and utilized in preliminary ELISAs to rank the performance of the immunogens obtained by various chemistries. We choose mice instead of rabbits as host animals to minimize both the time and expense of screening a large number of immunogens.

Immunogen Synthesis. In theory, the best immunogens should be obtained by attaching the linker group at the carbon backbone of the AAL toxin molecules, far from characteristic groups (Szurdoki et al., 1995). This approach would, however, require involved synthetic chemistry. Thus, we followed two basic strategies with simpler derivatizations. In the first case, the toxin molecule was linked to the nitrogen, opposite to the tricarballylic ester group (**N1**−**N5** conjugates, Figures 3−6). In this way, recognition of the tricarballylic moiety, the common major epitope in the toxin structures, would be emphasized. This approach seemed to be promising for devising class-selective assays as suggested by the results of the published fumonisin assays. However, it was not likely to be the optimal strategy for fumonisins due to the proposed cagelike conformation of these mycotoxins (Elissalde et al., 1995). The N-terminal part of TA exists in an unusual, rigid conformation even in aqueous solution (Oikawa et al., 1994). Our second approach, conjugation *via* a carboxyl group or groups (**C** conjugates, Figure 7), was designed to emphasize the antibody recognition of the N-terminal area of the TA molecule. For the syntheses of all immunogens, we used compound TA (Figure 1).

Derivatizations at the Amino Group. This approach includes modified glutaraldehyde couplings (**N1**, **N2**, Figure 3) and selective *N*-acylations (**N3**, **N4**, and **N5**, Figures 4−6). The classical, one-step glutaraldehyde ($CHO(CH_2)_3CHO$) conjugation of amino compounds to carrier proteins has been used widely (Reichlin, 1980) as well as for the preparation of most immunogenic FB_1-protein conjugates. In several syntheses, the method was combined with reductive stabilization by sodium borohydride (Azcona-Olivera et al., 1992; Fukuda et al., 1994). This chemistry preserves the basic character of the derivatized amino group, and the experimental protocol is straightforward. The conventional glutaraldehyde coupling has, however, a number of shortcomings, including precipitation of protein conjugates, loss of haptens due to oligomerization, poor control of the degree of substitution, and formation of highly complex mixtures (Zegers et al., 1990). Thus, improved, two-step versions of the glutaraldehyde method permitting controlled coupling have been developed (Tijssen, 1985; Zegers et al., 1990; Laman et al., 1991; Kamps-Holtzapple et al., 1994; Gabor et al., 1994). From these

Table 1. Sensitivities of Selected Competitive ELISAs Using Various Anti-TA Antibodies and TA-Derived Coating Antigens[a]

antiserum			IC_{50} (ppb)			
immunogen	mouse no.	coating antigen	TA	TB	TD	TE
N1−KLH	4	N3−BSA	114	60	130	34
N2−KLH	10	N3−BSA	266	149	184	97
N3−KLH	18	C−BSA	67	25	20	10
N4−OVA	57	C−BSA	32	30	28	13
N5−OVA	67	C−BSA	137	132	18	28
C−BSA	20	N2−KLH	87	136	242	30

[a] No significant cross-reactivity was observed for a number of structurally related compounds including FB_1 and HTA (IC_{50}s > 250 ppm). There was no significant inhibition with tricarballylic, citric, and *cis*-aconitic acids at 10 ppm and with DL-sphinganine at 20 ppm levels.

methods, we selected the mild and simple dialysis procedure (Zegers et al., 1990; Laman et al., 1991). The carrier proteins (BSA and KLH) were first activated by glutaraldehyde, excess reagent was removed, and compound TA was bound to the activated proteins by forming **N1** conjugates (Figure 3). The **N1** Schiff base is not the sole product of the condensation reaction; protein conjugates obtained by glutaraldehyde are often directly used as immunogens because some of the conjugates are hydrolytically stable (Reichlin, 1980; Zegers et al., 1990; Walt and Agayn, 1994). Therefore, **N1** conjugates were employed for immunization. However, aliquots of these conjugates were further stabilized by sodium cyanoborohydride ($NaBH_3CN$), furnishing the saturated **N2** derivatives (Figure 3). Sodium cyanoborohydride selectively reduces the C=N bond of Schiff bases in neutral solution (Geoghegan et al., 1981); thus, this reagent is compatible with the base-sensitive moieties (e.g., ester) of the derivatized compounds. (Posttreatment with sodium borohydride, often used for the same purpose, would have made the reaction mixture alkaline.) **N2** conjugates were also used as immunogens. The conjugates obtained by the two-step glutaraldehyde method without or with stabilization by sodium cyanoborohydride (**N1** and **N2**, respectively) resulted in markedly higher affinity antisera than those prepared by the one-pot glutaraldehyde/sodium borohydride procedure (Azcona-Olivera et al., 1992). There was no apparent improvement in the resulting ELISAs using **N2** immunogen as compared to **N1** (Table 1), implying that chemical stability of conjugate **N1** was not a problem. It is worth noting that the GA method without a further reduction step was employed for the synthesis of the immunogen in the development of the most sensitive fumonisin ELISA reported (Usleber et al., 1994).

For the synthesis of **N3** conjugates, TA toxin was conjugated with a new heterobifunctional cross-linker (**1**) to carrier proteins (BSA, KLH, Figure 4). The NHS ester of 4-(4-aminophenyl)butyric acid (**2**, Figure 8) was first prepared and used to acylate the aliphatic amine TA toxin. The aromatic amino group of the linker was then bound to proteins *via* diazotation and azo coupling (Fujiwara et al., 1990). The selective acylation of the TA molecule without extensive oligomerization of the aromatic amine cross-linker was feasible because the aromatic amino groups are poor nucleophiles and only weakly reactive toward NHS esters (Denny and Blobel, 1984; Fujiwara et al., 1990; Battaglini et al., 1994). Model experiments with 1-butylamine (**3**) mimicking TA (Figure 8) were in agreement with these literature reports. The 1-butylamide (**4**) of acid **1** was the main product, and the corresponding amide (**5**) of the dimer of the starting material (**1**) was detected as a minor contamination by MS (Figure 8).

In the next two syntheses, carrier proteins were thiolated by routine procedures. Heterobifunctional cross-linkers with the thiol-specific maleimido group were then applied to secure **N4** and **N5** conjugates (Figures 5 and 6). Proteins were thiolated by successive treatments with SAMSA and hydroxylamine (Tijssen, 1985; Fujiwara et al., 1981); TA toxin was then coupled to the thiol-containing protein derivatives by MHS to produce **N4** conjugates (Figure 5). In a recent comparative study with several heterobifunctional cross-linking agents, MHS was the reagent of choice for the preparation of immunogens due to the chemical stability of the protein conjugates and the resulting low linker-specific antibody levels (Peeters et al., 1989). A reaction sequence similar to one used in a fumonisin-ELISA development (Kamps-Holtzapple et al., 1993; Elissalde et al., 1995) resulted in **N5** conjugates (Figure 6).

Derivatization at the Carboxyl Group(s). A second objective was to develop a simple method for preparing conjugates with a significant fraction of coupling through the carboxyl group(s) of the toxin molecule (**C**, Figure 7). Water-soluble carbodiimides, like EDC, are employed often for the conjugation of ligands with carboxyl and/or amino functional groups (e.g., peptides, toxins) to proteins and other carrier macromolecules through the formation of amide bonds (Briand et al., 1985; Smith and Kitts, 1995). This approach involves a simple coupling procedure; however, it has several disadvantages including extensive intramolecular cross-linking of the carrier protein, formation of polymers containing protein and ligand monomers, and loss of reagents due to precipitation of side products with high molecular weight. In addition, the use of immunogens synthesized by this method frequently results in strong antibody responses against unwanted new antigenic determinants on the conjugated proteins (Briand et al., 1985; Szurdoki et al., 1995). The yields of the attachment of ligands to carrier macromolecules are occasionally low and variable (Sehgal and Vijay, 1994). Addition of NHS (Sehgal and Vijay, 1994) or sulfo-NHS (Staros et al., 1986) to the aqueous reaction mixtures of the carbodiimide-mediated conjugations resulted in significantly improved yields.

Simultaneous incubation of TA toxin (TA) and the carrier protein under these improved cross-linking conditions still might result in polymerization of the hapten molecule and the protein and no preference for the derivatization of TA through its carboxyl group(s). A literature search revealed that two-step procedures carried out in aqueous solutions were recently devised to minimize side reactions in similar conjugations (Klibanov et al., 1989; Grabarek and Gergely, 1990; Varadarajan and Hawthorne, 1991; Bedel-Cloutour et al., 1991; Lewis et al., 1994). Following these leads, TA was first treated with EDC and sulfo-NHS under mildly acidic conditions with brief incubation at low temperature (Moffett et al., 1993; Sehgal and Vijay, 1994) to minimize oligomerization. Coupling with proteins was then carried out in alkaline bicarbonate buffer (Figure 7). Phosphate was not used to buffer this aqueous reaction mixture because phosphates were recently shown to react with carbodiimides rendering them useless for condensation (Moffett et al., 1993). Conjugation of the carboxyl group(s) of the hapten (TA) to proteins also may have resulted in acylation of the amino group and formation of oligomers of TA as the

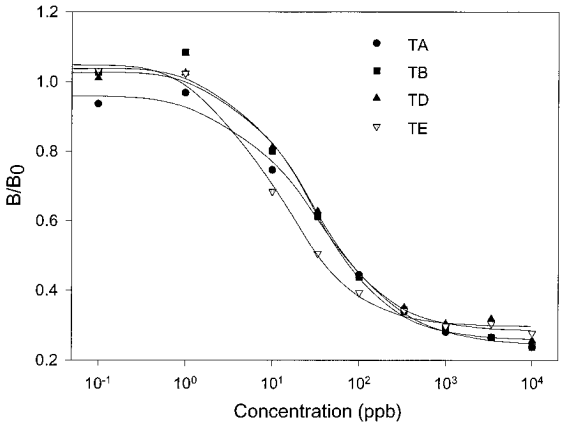

Figure 9. Standard curves for selected AAL toxins (TA, TB, TD, TE). Data were obtained by the ELISA system involving mouse antiserum no. 57 (bleed no. 3), raised against conjugate **N4**−OVA, and coating antigen **C**−BSA (cf. Table 1). The average and maximum relative standard deviations of four independent well replicates for each data point were 6.5 and 13%, respectively. Absorbance values at zero concentration were in the range of 0.55−0.7.

cross-reactivity pattern of the antiserum raised against the **C**−BSA conjugate appears to indicate (see below; Table 1). Nevertheless, **C**−BSA conjugate (Figure 7) was one of the best immunogens and plate coating antigens (Table 1). Further investigation with this type of coupling reaction is in progress.

The TA structure does not have chromophores with strong absorbance in the suitable regions of the UV−visible spectrum. Therefore, no attempts were made to verify the conjugation reactions. Most conjugates used as coating antigens, however, were recognized by heterologous antisera.

ELISA Development. Some immunogens elicited high-titer antisera in mice after the first boost, and titers remained high throughout the immunization period. Preliminary competitive ELISAs in the coating antigen format, developed for screening antisera with adequate titers, provided information about the immunogenicity of the synthetic antigens and the performance of ELISAs in the early stages of the immunization protocol. The interim data obtained on the first few immunogens prompted us to synthesize further conjugates. In most cases, blood samples taken after the second boost served for the development of the assays used to characterize the antisera and evaluate the immunogens. In general, ELISAs with appropriate sensitivities and selectivities were obtained in this preliminary study (Table 1).

Our selected ELISA results, summarized in Table 1, demonstrate that AAL toxins are detected at parts per billion levels with assay systems based on immunogens representing all conjugation methods. The most sensitive ELISAs have detection limits in the low-ppb range (Figure 9). The dose−response curves obtained with the AAL compounds TA, TB, TD, and TE confirmed the detection of the *Alternaria* toxins as a class. The high sensitivity for TE, a toxin with an acetylated amino group (Figure 1), is likely due to the recognition of the linker groups in the immunogens attached to the nitrogen of TA. It also is possible that the conformation of TA in conjugates with the linker attached at the nitrogen of this toxin more closely resembles the conformation of TE than that of the parent TA. No significant cross-reactivities were displayed by hydrolyzed TA (HTA, $R_1 = R_2 = R_5 = H$, $R_3 = R_4 = OH$, Figure 1), FB_1, DL-sphinganine, and tricarballylic, citric, and *cis*-aconitic acids (Table 1, Figure 2).

Although our assay systems are not fully optimized, it appears that **N3**, **N4**, and **C** are novel chemistries that deserve attention (Table 1). The **C**−BSA conjugate synthesized by the activation of the carboxyl group(s) showed good performance as immunogen and when it was used for plate coating. As expected, antiserum raised by this conjugate had the strongest preference for TA versus TB and TD due to enhanced recognition of the N-terminal area of the target molecule. The high cross-reactivity for TE seems to indicate that **C**−BSA probably contains not only conjugates with linkage through the carboxyl group(s) but also ones with the amino group derivatized and with oligomers of TA.

Use of the **N4**−OVA conjugate (Figure 5), a 6-(*N*-maleimido)hexanoate derivative, as immunogen resulted in the most sensitive assay for TA (Table 1, Figure 9). Interestingly, although the **N3**−KLH, **N4**−OVA, and **N5**−OVA conjugates (Figures 4−6) have similar linker groups, the **N4**−OVA immunogen showed the best performance. While the structure of **N4** conjugates is characterized by a 12-atom long linker, the spacer group in **N5** protein derivatives is somewhat larger (16 atoms long) and contains an additional benzene ring. The linker group of **N3** conjugates (10 atoms long) shares the aralkyl moiety, attached directly to the toxin molecule, with the **N5** structure. The ideal linker group in the immunogen conjugate of a small molecule usually is a medium-sized straight chain (length: about 4−6 carbon atoms) (Szurdoki et al., 1995). It has been suggested that inclusion of a highly immunogenic group like an aromatic ring may cause elevated linker-specific antibody levels which would diminish the performance of the resulting ELISA (Peeters et al., 1989; Szurdoki et al., 1995). However, recently it has been observed that application of longer bridging groups (Kamps-Holtzapple et al., 1994; Elissalde et al., 1995) in the immunogen structure, like that of conjugate **N5**, provided fair to high sensitivity immunoassays for molecules with dimensions and complexity similar to those of TA. The fact that our most sensitive ELISA was obtained with the MHS linker (conjugate **N4**) supports results by Peeters et al. (1989) and also follows expectation based on the structures of the bridging groups when compared to performances of immunogens **N3** and **N5** with linkers including benzene rings. It is not clear, however, why immunogen **N4** worked better than the glutaraldehyde-derived conjugates (**N1** and **N2**; Figure 3), although a similar observation has been reported (Kamps-Holtzapple et al., 1994). Different densities and steric orientations of haptens on the carrier protein surfaces as well as variation in immune response among individual animals also could be responsible for unexpected results. It is usually rewarding to investigate several conjugation methods and/or haptens during immunoassay development for difficult targets, and it is often hard to predict the rank-order of the immunogens (Briand et al., 1985; Kamps-Holtzapple et al., 1994; Szurdoki et al., 1995). The conjugates that elicited the best antisera are candidate immunogens to produce further polyclonal antisera and monoclonal antibodies. Our synthesis principles have been applied to prepare immunogens from other AAL toxins and fumonisins.

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LITERATURE CITED

Abbas, H. K.; Tanaka, T.; Duke, S. O.; Porter, J. K.; Wray, E. M.; Hodges, L.; Sessions, A. E.; Wang, E.; Merrill, A. H., Jr.; Riley, R. T. Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. *Plant Physiol.* **1994**, *106*, 1085−1093.

Azcona-Olivera, J. I.; Abouzied, M. M.; Plattner, R. D.; Pestka, J. J. Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2, and B_3. *J. Agric. Food Chem.* **1992**, *40*, 531−534.

Battaglini, F.; Koutroumanis, M.; English, A. M.; Mikkelsen, S. R. Targeting glucose oxidase at aspartate and glutamate residues with organic two-electron redox mediators. *Bioconjugate Chem.* **1994**, *5*, 430−435.

Bedel-Cloutour, C. H.; Maneta-Peyret, L.; Pereyre, M.; Bezian, J.-H. Synthesis of a monoclonal antibody-indium-111-porphyrin conjugate. *J. Immunol. Methods* **1991**, *144*, 35−41.

Bottini, A. T.; Bowen, J. R.; Gilchrist, D. G. Phytotoxins. II. Characterization of a phytotoxic fraction from *Alternaria alternata* f. sp. *lycopersici*. *Tetrahedron Lett.* **1981**, *22*, 2723−2726.

Boyle, C. D.; Kishi, Y. Absolute configuration at the tricarballylic acid moieties of fumonisin B_1 and AAL toxin T_A. *Tetrahedron Lett.* **1995**, *36*, 5695−5698.

Boyle, C. D.; Harmange, J.-C.; Kishi, Y. Novel structure elucidation of AAL toxin T_A backbone. *J. Am. Chem. Soc.* **1994**, *116*, 4995−4996.

Briand, J. P.; Muller, S.; Van Regenmortel, M. H. V. Synthetic peptides as antigens: pitfalls of conjugation methods. *J. Immunol. Methods* **1985**, *78*, 59−69.

Caldas, E. D.; Jones, A. D.; Ward, B.; Winter, C. K.; Gilchrist, D. G. Structural characterization of three new AAL toxins produced by *Alternaria alternata* f. sp. *lycopersici*. *J. Agric. Food Chem.* **1994**, *42*, 327−333.

Caldas, E. D.; Jones, A. D.; Winter, C. K.; Ward, B.; Gilchrist, D. G. Electrospray ionization mass spectrometry of sphinganine analog mycotoxins. *Anal. Chem.* **1995**, *67*, 196−207.

Chu, F. S.; Huang, X.; Maragos, C. M. Production and characterization of anti-idiotype and anti-anti-idiotype antibodies against fumonisin B_1. *J. Agric. Food Chem.* **1995**, *43*, 261−267.

Denny, J. B.; Blobel, G. ^{125}I-labeled crosslinking reagent that is hydrophilic, photoactivable, and cleavable through an azo linkage. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 5286−5290.

Elissalde, M. H.; Kamps-Holtzapple, C.; Beier, R. C.; Plattner, R. D.; Rowe, L. D.; Stanker, L. H. Development of an improved monoclonal-based enzyme linked immunosorbent assay for fumonisin B_{1-3} and the use of molecular modeling to explain observed detection limits. *Food Agric. Immunol.* **1995**, *7*, 109−122.

Freedman, L. D.; Doak, G. O. Arsenoso derivatives of phenyl-substituted fatty acids. *J. Am. Chem. Soc.* **1949**, *71*, 779−780.

Fujiwara, K.; Yasuno, M.; Kitagawa, T. Novel preparation method of immunogen for hydrophobic hapten, enzyme immunoassay for daunomycin and adriamycin. *J. Immunol. Methods* **1981**, *45*, 195−203.

Fujiwara, K.; Matsumoto, N.; Kitagawa, T.; Inouye, K. The use of *N*-(aminobenzoyloxy)succinimide as a two-level heterobifunctional agent for the preparation of hapten-protein conjugates. *J. Immunol. Methods* **1990**, *134*, 227−235.

Fukuda, S.; Nagahara, A.; Kikuchi, M.; Kumagai, S. Preparation and characterization of anti-fumonisin monoclonal antibodies. *Biosci. Biotech. Biochem.* **1994**, *58*, 765−767.

Gabor, F.; Wollmann, K.; Theyer, G.; Haberl, I.; Hamilton, G. *In vitro* antiproliferative effects of albumin-doxorubicin conjugates against Ewing's sarcoma and peripheral neuroectodermal tumor cells. *Anticancer Res.* **1994**, *14*, 1943−1950.

Geoghegan, K. F.; Cabacungan, J. C.; Dixon, H. B. F.; Feeney, R. E. Alternative reducing agents for reductive methylation of amino groups in proteins. *Int. J. Pept. Protein Res.* **1981**, *17*, 345−352.

Gilchrist, D. G.; Ward, B.; Moussato, V.; Mirocha, C. J. Genetic and physiological response to fumonisin and AAL-toxin by intact tissue of a higher plant. *Mycopathologia* **1992**, *117*, 57−64.

Grabarek, Z.; Gergely, J. Zero-length crosslinking procedure with the use of active esters. *Anal. Biochem.* **1990**, *185*, 131−135.

Kamps-Holtzapple, C.; Carlin, R. J.; Sheffield, C.; Kubena, L.; Stanker, L.; DeLoach, J. R. Analysis of hapten-carrier protein conjugates by nondenaturing gel electrophoresis. *J. Immunol. Methods* **1993**, *164*, 245−253.

Kamps-Holtzapple, C.; Stanker, L. H.; DeLoach, J. R. Development of a monoclonal antibody-based ELISA for the anthelmintic hygromycin B. *J. Agric. Food Chem.* **1994**, *42*, 822−827.

Klibanov, A. L.; Slinkin, M. A.; Torchilin, V. P. Conjugation of proteins with chelating polymers via water-soluble carbodiimide and *N*-hydroxysulfosuccinimide. *Appl. Biochem. Biotechnol.* **1989**, *22*, 45−58.

Laman, J. D.; van den Eertwegh, A. J. M.; Deen, C.; Vermeulen, N.; Boersma, W. J. A.; Claassen, E. Synthetic peptide conjugates with horseradish peroxidase and β-galactosidase for use in epitope-specific immunocytochemistry and ELISA. *J. Immunol. Methods* **1991**, *145*, 1−10.

Lewis, M. R.; Raubitschek, A.; Shively, J. E. A facile, water-soluble method for modification of proteins with DOTA. Use of elevated temperature and optimized pH to achieve high specific activity and high chelate stability in radiolabeled immunoconjugates. *Bioconjugate Chem.* **1994**, **5**, 565−76.

Maragos, C. M. Capillary zone electrophoresis and HPLC for the analysis of fluorescein isothiocyanate-labeled fumonisin B_1. *J. Agric. Food Chem.* **1995**, *43*, 390−394.

Merrill, A. H., Jr.; Wang, E.; Gilchrist, D. G.; Riley, R. T. Fumonisins and other inhibitors of *de novo* sphingolipid biosynthesis. *Adv. Lipid Res.* **1993**, *26*, 215−234.

Mirocha, C. J.; Gilchrist, D. G.; Shier, W. T.; Abbas, H. K.; Wen, Y.; Vesonder, R. F. AAL toxins, fumonisins (biology and chemistry) and host-specificity concepts. *Mycopathologia* **1992**, *117*, 47−56.

Moffett, J. R.; Namboodiri, M. A. A.; Neale, J. H. Enhanced carbodiimide fixation for immunohistochemistry: application to the comparative distributions of *N*-acetylaspartylglutamate and *N*-acetylaspartate immunoreactivities in rat brain. *J. Histochem. Cytochem.* **1993**, *41*, 559−570.

Nelson, P. E.; Desjardins, A. E.; Plattner, R. D. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annu. Rev. Phytopathol.* **1993**, *31*, 233−252.

Norred, W. P.; Voss, K. A. Toxicity and role of fumonisins in animal diseases and human esophageal cancer. *J. Food Prot.* **1994**, *57*, 522−527.

Oikawa, H.; Matsuda, I.; Kagawa, T.; Ichihara, A; Kohmoto, K. Absolute configuration of main chain of AAL-toxins. *Tetrahedron* **1994**, *50*, 13347−13368.

Peeters, J. M.; Hazendonk, T. G.; Beuvery, E. C.; Tesser, G. I. Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J. Immunol. Methods* **1989**, *120*, 133−143.

Pestka, J. J.; Azcona-Olivera, J. I.; Plattner, R. D.; Minervini, F.; Doko, M. B.; Visconti, A. Comparative assessment of fumonisin in grain-based foods by ELISA, GC-MS, and HPLC. *J. Food Prot.* **1994**, *57*, 169−172.

Reichlin, M. Use of glutaraldehyde as a coupling agent for proteins and peptides. In *Methods in Enzymology*, Van Vunakis, H., Langone, J. J., Eds.; Academic Press: New York, 1980; Vol. 70, pp 159−165.

Riley, R. T.; Voss, K. A.; Yoo, H.-S.; Gelderblom, W. C. A; Merrill, A. H., Jr. Mechanism of fumonisin toxicity and carcinogenesis. *J. Food Prot.* **1994**, *57*, 638−645.

Rodbard, D. Mathematics and statistics of ligand assay: an illustrated guide. In *Ligand Assay*; Langan, J., Clapp, J. J., Eds.; Masson Publishing: New York, 1981; pp 45−99.

Sehgal, D.; Vijay, I. K. A method for the high efficiency of water-soluble carbodiimide-mediated amidation. *Anal. Biochem.* **1994**, *218*, 87−91.

Shelby, R. A.; Rottinghaus, G. E.; Minor, H. C. Comparison of thin-layer chromatography and competitive immunoassay methods for detecting fumonisin on maize. *J. Agric. Food Chem.* **1994**, *42*, 2064−2067.

Siler, D. J.; Gilchrist, D. G. Determination of host-selective phytotoxins from *Alternaria alternata* f. sp. *lycopersici* as their maleyl derivatives by high-performance liquid chromatography. *J. Chromatogr.* **1982**, *238*, 167−173.

Smith, D. S.; Kitts, D. D. Enzyme Immunoassay for the Determination of Domic Acid in Mussel Extracts. *J. Agric. Food. Chem.* **1995**, *43*, 367−371.

Staros, J. V.; Wright, R. W.; Swingle, D. M. Enhancement by *N*-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal. Biochem.* **1986**, *156*, 220−222.

Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. Important factors in hapten design and enzyme-linked immunosorbent assay development. In *New Frontiers of Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 39−63.

Szurdoki, F.; Trousdale, E.; Gee, S. J.; Ward, B.; Hammock, B. D.; Gilchrist, D. G. Development of an enzyme immunoassay for *Alternaria alternata* f.sp. *lycopersici* toxins. In *Immunoassays for Residue Analysis: Food Safety*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 330−340.

Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A. The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **1992**, *117*, 3−9.

Tijssen, P. *Practice and Theory of Enzyme Immunoassays*; Laboratory Techniques in Biochemistry and Molecular Biology 15; Burdon, R. H., van Knippenberg, P. H., Eds.; Elsevier: Amsterdam, The Netherlands, 1985.

Usleber, E.; Straka, M.; Terplan, G. Enzyme immunoassay for fumonisin B_1 applied to corn-based food. *J. Agric. Food Chem.* **1994**, *42*, 1392−1396.

Varadarajan, A; Hawthorne, M. F. Novel carboranyl amino acids and peptides: reagents for antibody modification and subsequent neutron-capture studies. *Bioconjugate Chem.* **1991**, *2*, 242−253.

Walt, D. R.; Agayn, V. I. The chemistry of enzyme and protein immobilization with glutaraldehyde. *Trends Anal. Chem.* **1994**, *13*, 425−430.

Wang, H.; Jones, C.; Ciacci-Zanella, J.; Holt, T.; Gilchrist, D. G.; Dickman, M. B. Fumonisins and *Alternaria alternata lycopersici* toxins: sphinganine analog mycotoxins induce apoptosis in monkey kidney cells. *Proc. Natl. Acad. Sci. U.S.A.* **1996a**, *93*, 3461−3465.

Wang, H.; Li, J.; Bostock, R. M.; Gilchrist, D. G. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* **1996b**, *8*, 375−391.

Ware, G. M.; Umrigar, P. P.; Carman, A. S., Jr.; Kuan, S. S. Evaluation of Fumonitest immunoaffinity columns. *Anal. Lett.* **1994**, *27*, 693−715.

Wu, W.-I; McDonough, V. M.; Nickels, J. T., Jr.; Ko, J.; Fischl, A. S.; Vales, T. R.; Merrill, A. H., Jr.; Carman, G. M. Regulation of lipid biosynthesis in *Saccharomyces cerevisiae* by fumonisin B_1. *J. Biol. Chem.* **1995**, *270*, 13171−13178.

Zegers, N. D.; Gerritse, K.; Deen, C.; Boersma, W.; Claassen, E. An improved conjugation method for controlled covalent coupling of synthetic peptides to proteins using glutaraldehyde in a dialysis method. *J. Immunol. Methods* **1990**, *130*, 195−200.

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