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Immunoassays for Residue Analysis

Food Safety

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Chapter 25

Development of an Enzyme Immunoassay for *Alternaria alternata* f.sp. *lycopersici* Toxins

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AAL toxins and fumonisins are sphinganine analog mycotoxins secreted by the fungal pathogens *Alternaria alternata* f.sp. *lycopersici* and *Fusarium moniliforme*, respectively. Consumption of fumonisin-contaminated maize has been reported to cause several animal diseases and has been correlated with high incidence of human esophageal cancer. Recent studies also demonstrate that both fumonisins and AAL compounds are phytotoxic to tomato, cytotoxic to cultured mammalian cells, and inhibit ceramide synthase in animals. Preliminary evidence indicates that AAL toxins are present in field-grown tomatoes, destined for processing, which show signs of the blackmold disease. An enzyme-linked immunosorbent assay (ELISA) would provide a rapid and inexpensive analytical tool for screening large numbers of food samples for AAL toxins. We report here the development of a class-selective ELISA for detection of AAL toxins at ppb to low ppm levels. Mice were inoculated with various protein conjugates of AAL compound TA and the elicited polyclonal antibodies were used for assay development. No cross-reactivities were found with sphinganine, the structurally related sphingoid base, or with fumonisin B_1. The sensitivity and selectivity of the assay produced in this preliminary study indicate a high potential for development of ELISAs for the determination of AAL toxins in food and feed samples.

Gilchrist and Grogan (*1*) demonstrated that the fungal pathogen *Alternaria alternata* f.sp. *lycopersici* produces AAL toxins (Figure 1). These toxins are the primary chemical determinants of Alternaria stem canker disease of tomato cultivars homozygous-recessive for the *Asc* gene (*asc/asc*) (*2*). Another class of mycotoxins, the fumonisins (Figure 2), are secreted by several species of *Fusarium* including *Fusarium moniliforme* Sheldon, an economically important pathogen of maize and other grains (*3*, *4*). In tomato, the *Asc* gene regulates sensitivity to both AAL toxins and the fumonisins (*2*).

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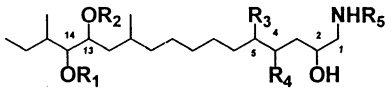
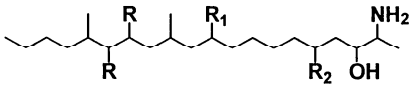


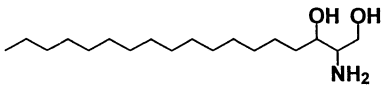
Toxin	R_1	R_2	R_3	R_4	R_5
TA_1	H	R	OH	OH	H
TA_2	R	H	OH	OH	H
TB_1	H	R	H	OH	H
TB_2	R	H	H	OH	H
TC_1	H	R	H	H	H
TC_2	R	H	H	H	H
TD_1	H	R	H	OH	$COCH_3$
TD_2	R	H	H	OH	$COCH_3$
TE_1	H	R	H	H	$COCH_3$
TE_2	R	H	H	H	$COCH_3$

R-: $HO_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO$-

Figure 1. Structures of AAL toxins.



Toxin	R	R_1	R_2
FB_1	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	OH	OH
FB_2	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	H	OH
FB_3	$O_2C\text{-}CH_2\text{-}CH(CO_2H)\text{-}CH_2\text{-}CO_2H$	OH	H



Sphinganine

Figure 2. Structures of fumonisins and sphinganine.

AAL Toxins

Isolation of the five main structural congeners of AAL toxin (TA, TB, TC, TD, and TE) and elucidation of their gross chemical structures have been reported by Bottini *et al.* (*5*) and Caldas *et al.* (*6*, *7*). Each toxin fraction is an isomeric-mixture of an aminopolyol backbone esterified by a terminal carboxyl group of tricarballylic acid at the C-13 or C-14 hydroxyl groups (Figure 1). The absolute configuration of the main chain of compound TA was determined independently by two groups (*8*, *9*). Recently, the configuration of the tricarballylic acid residue was also reported (*10*). The NMR studies and MM2 calculations indicated the presence of strong hydrogen bonds between NH → 2-O and 4-OH → 5-O positions which stabilizes the unique conformation of the segment around the amino-group of the TA molecule even in aqueous solution (*9*).

Structural and Biochemical Similarity of AAL Toxins and Fumonisins

Fumonisins (e.g., FB_1, Figure 2) (*11*–*13*) are structurally related to AAL compounds (Figure 1). Compounds FB_1, FB_2, and FB_3 are diesters of a family of aminopolyols with both hydroxyl groups at C-14 and C-15 esterified with the terminal carboxyl group of tricarballylic acid (Figure 2). Both the fumonisins and AAL toxins bear structural similarity to sphingoid bases such as sphinganine (Figure 2). The most recent stereochemical analyses (*8*, *10*, *14*) demonstrate that FB_1 and TA have identical configurations at all common stereocenters throughout the aminopolyol backbones and tricarballylic ester side chains.

In animals, the fumonisins were shown to cause a variety of serious or fatal diseases. Although FB_1 does not appear to be genotoxic, it is carcinogenic in rat liver (*15*), and exhibits marked activity as a cancer promoter (*16*, *17*). Leukoencephalomacia (ELEM) in equines, porcine pulmonary edema (PPE), hepatotoxicity and nephrotoxicity in rats, and hepatocellular hyperplasia in turkey poults are animal disease conditions produced by the fumonisins (*4*, *15*, *17*–*22*). Epidemiological data also link the consumption of *F. moniliforme*-contaminated maize with a high incidence of human esophageal cancer in the Transkei region of southern Africa (*23*). Both fumonisin FB_1 and AAL-toxin TA were found to be cytotoxic in mammalian cell cultures (*24*, *25*) and phytotoxic to susceptible tomato varieties (*asc/asc*) (*2*, *26*).

Both the AAL toxins and the fumonisins are potent inhibitors of sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase), and thereby disrupt sphingolipid metabolism, which may account for a number of their biological effects (*15*, *27*–*31*). It is well established that sphingolipids are of great importance in cellular signaling systems, and interference of fumonisins and AAL toxins with sphingolipid metabolism may result in cellular deregulation (*15*). Exposure to these toxins leads to elevated concentrations of free sphingoid bases (e.g., sphinganine, Figure 2) in both animals (*15*) and plants (*29*), which correlates with cell and tissue damage. Fumonisins contaminate maize and maize-based human and animal foodstuffs usually in low ppb to low ppm levels worldwide (*4*, *7*, *32*–*34*). Consumption of foodstuffs with relatively high concentrations of fumonisins involves potential health hazards (*32*).

To our knowledge, no detailed animal toxicity data are available for *Alternaria* toxins. Nevertheless, the structural similarity and common mode of biochemical action of AAL compounds and fumonisins are sufficient reason for concern about the presence of AAL toxins in the food chain (*2*, *6*, *25*). Risk assessment for both groups of compounds, including studies on situations where they occur together or with other natural toxins (*35*) in foods is warranted. Several investigations on the combined toxicological effects and interactions have already been performed (*19*, *20*, *36*).

Analytical Methods for AAL Compounds and Fumonisins

Field tomato samples and commercial tomato-based food products are often contaminated with AAL toxins in the low ppb to low ppm concentration range (Gilchrist, D. G., University of California, Davis, unpublished results). Currently, only instrumental analyses such as high-performance liquid chromatography (HPLC) (*37*) and electrospray ionization-mass spectrometry (ESI-MS) (*7*) are available for quantitative determination of AAL compounds in food. An enzyme-linked immunosorbent assay (ELISA) would provide scientists and regulatory agencies with a rapid and inexpensive analytical method for large-scale monitoring of *Alternaria* toxins. A further advantage of the immunochemical analysis would be the potential to develop an immunoaffinity cleanup method using the anti-AAL antibodies which could remarkably enhance instrumental analyses as it was demonstrated with the commercial immunoaffinity columns for fumonisins (*38*–*40*).

Thin layer chromatography (*4*, *41*) and instrumental methods like HPLC, ESI-MS, gas chromatography-MS (*4*, *7*, *32*, *40*, *42*–*44*), capillary electrophoresis (CE) (*45*) and CE-ESI-MS (*46*) are used most often for the analysis of fumonisins. These analytical methods are highly sensitive and selective; however, they have a number of shortcomings such as need for expensive apparatus, trained personnel, and laborious sample preparation. Hence, these methods are not useful for low-cost screening of large numbers of samples and for on-site analyses. In an attempt to address these limitations, immunoassays have recently been devised for the detection of ppb to ppm concentrations of fumonisins (*33*, *41*, *42*, *47*–*52*). These assays are class-selective for the fumonisins, they do not cross-react with the AAL toxins.

Methods

Modified procedures (*53*–*55*) were used for conjugation of TA toxin to carrier proteins to obtain immunogens and coating antigens (see below). Polyclonal mouse antisera were raised following standard protocols (*53*, *56*). Indirect ELISA in the coating antigen format was similar to that of Bekheit *et al.* (*57*). Briefly, the mixture of the competitor and the mouse anti-TA antibody was incubated in the coated wells. The wells were then treated with enzyme-labeled goat anti-mouse antibody, and finally with the chromogen enzyme-substrate. Photometric detection of the colored product, generated by the enzyme, allowed to obtain standard curves with the analytes. The full account of our studies including experimental details and results with further immunogens will be given elsewhere.

Results and Discussion

Synthesis of Immunogens. Most of the fumonisin-immunoassays are based on immunogens prepared by the conventional glutaraldehyde (GA) coupling (cf. Figure 3) (*33*, *47*, *48*, *51*). This one-step method is combined with the reduction of the formed Schiff base by sodium borohydride to stabilize the fumonisin-protein conjugate in several procedures (*47*, *48*, *51*). Another approach by Elissalde *et al.* (*49*) used a more elaborate synthesis: carrier proteins were thiolated with 2-iminothiolane (2-IMT), and FB_1 was then conjugated to these protein derivatives by means of sulfosuccinimidyl 4-(4-(*N*-maleimido)phenyl)butyrate (sulfo-SMPB) (*54*), a heterobifunctional cross-linker (cf. Figure 4).

In the beginning of our studies, we failed to elicit useful antisera with protein-AAL toxin conjugates obtained by a one-step GA method (Figure 3) combined with sodium borohydride-reduction, a procedure similar to what was used in the first published fumonisin-ELISAs (*47*, *48*). In this and the following conjugation reactions, AAL toxin TA was used as the ligand. The one-step GA method, in which the ligand, protein, and GA are mixed, is poorly controlled and usually results in a complex mixture of polymerized products. However, an improved, two-step GA coupling (*53*), permitting formation of better defined, stable conjugates without reductive post-treatment, has been successfully applied for the synthesis of highly immunogenic protein-toxin conjugates (Figure 3). In this procedure, the carrier protein is first treated with an excess of GA, which is eliminated before the reaction with the toxin. The application of sulfo-SMPB (*55*), the heterobifunctional cross-linker used previously in the development of a fumonisin-assay (*49*, *54*), also led to useful immunogens (Figure 4).

Antibody Production and ELISA Development. Polyclonal mouse antisera were raised against both sets of protein conjugates using routine protocols (*53*, *56*). Heterologous ELISAs in the immobilized antigen format were developed with the resulting high-titer antisera in the early phases of the immunization schedules. On the basis of these preliminary competitive immunoassay data, further immunogens were designed. (The results obtained with these new synthetic immunogens will be presented elsewhere.) By using several mice for each conjugate, we were able to evaluate our coupling methods with reduced cost and time. We found this approach to be more feasible than obtaining rabbit polyclonal antisera when used with a number of immunogens.

Assays using the best antiserum-coating antigen combinations detected the AAL compounds at ppb to low ppm concentrations and displayed class-selectivity for the *Alternaria* toxins. The performance of one of our ELISAs is illustrated in Table I and Figure 5. The high sensitivity (Table I and Figure 5) for TE, a containing an *N*-acetyl-group (Figure 1), is likely due to recognition of the linker attached to the amino-group of TA in the structure of the immunogen conjugate (TA-GA-KLH, see Figures 1 and 3). No cross-reactivity with structurally similar natural products (FB_1 and sphinganine, see Figure 2) was found.

In this study, we devised an ELISA for the selective detection of the entire set of AAL toxins. The sensitivity and selectivity of the assay hold promise for the analysis AAL toxins in food samples. The best immunogens will be used to develop

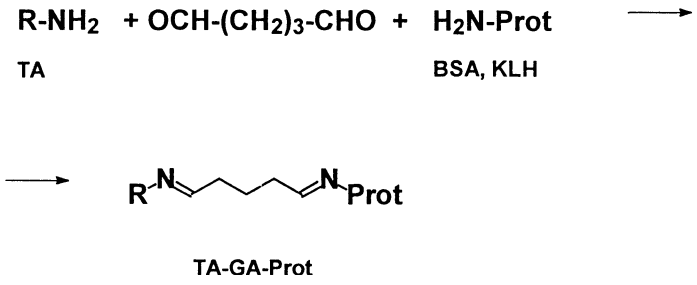


Figure 3. Glutaraldehyde coupling. Carrier proteins: BSA, KLH.

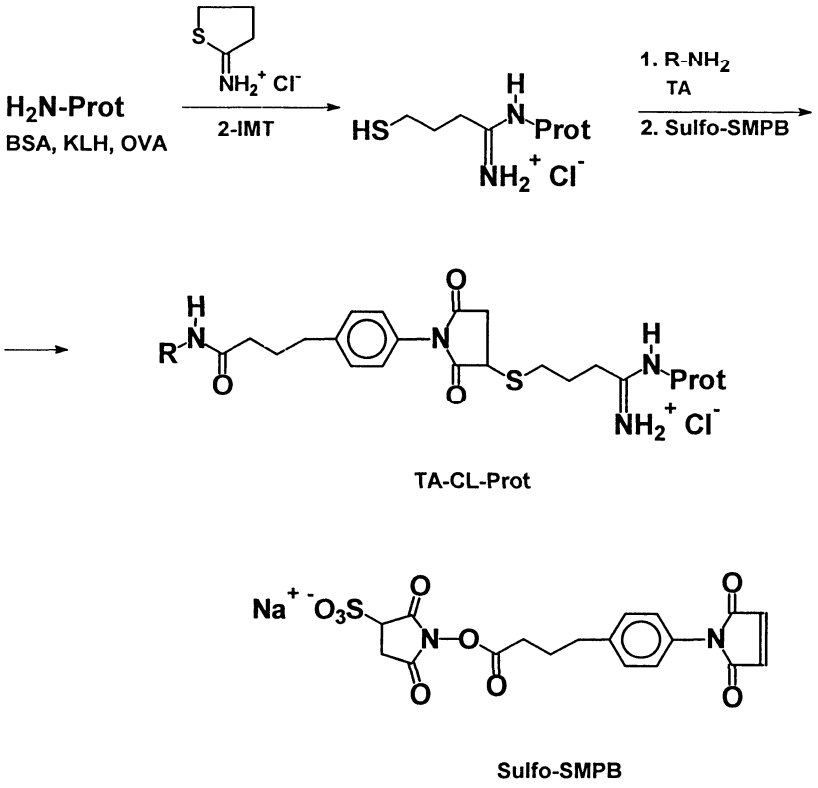


Figure 4. Synthesis of conjugates by protein thiolation with 2-IMT and then by using heterobifunctional cross-linker sulfo-SMPB. Carrier proteins: BSA, KLH, OVA.

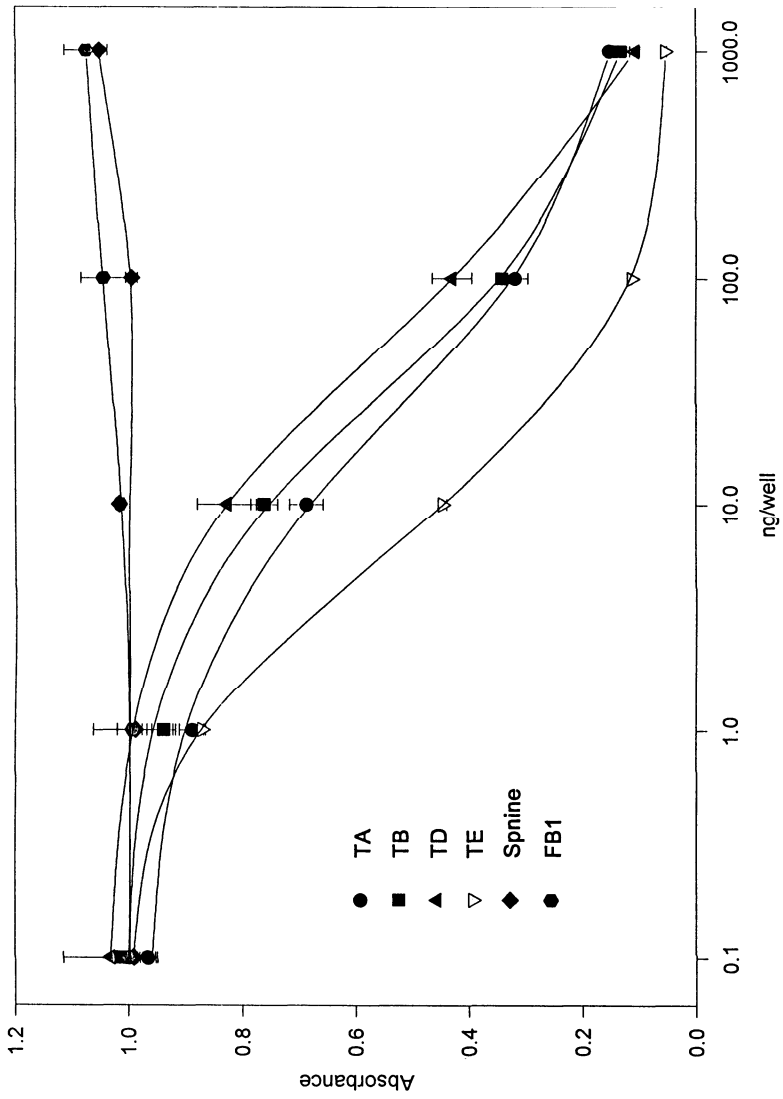


Figure 5. Standard curves obtained by the ELISA system involves mouse antiserum raised against TA-GA-KLH ard coating antigen TA-CL-OVA (cf. Table 1). Concentration: ng/well = ng in 50 µL. Spnine: sphinganine.

additional polyclonal antisera and monoclonal antibodies. Studies are currently underway with novel conjugation chemistries, syntheses of immunogens from other AAL toxins and fumonisins, ELISA development for fumonisins, and with real samples.

Table I. Competitive ELISA results with AAL Toxins.

		TA	TB	TD	TE
IC_{50}[a,b]	(ng/well)	24.5	33.0	65.7	6.9
IC_{50}	(ppb)	490	660	1314	138
CR[c]	(%)	100	74	37	355

[a]ELISA system involves mouse antiserum raised against TA-GA-KLH and coating antigen TA-CL-OVA. TA-GA-KLH was obtained by the two-step GA coupling (Figure 3). TA-CL-OVA was synthesized by protein thiolation and then by using sulfo-SMPB, a heterobifunctional cross-linker (Figure 4). No competition was observed with FB_1 and sphinganine (Figure 2).
[b]IC_{50}: analyte concentration required for 50% inhibition.
[c]CR: cross-reactivity (%) = 100 x IC_{50}[TA]/IC_{50}[analyte].

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