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Dipstick Enzyme Immunoassay To Detect *Fusarium* T-2 Toxin in Wheat

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A dipstick enzyme immunoassay for the rapid detection of *Fusarium* T-2 toxin in wheat was developed. An Immunodyne ABC membrane was precoated with rabbit anti-mouse immunoglobulins. After the strips were immersed in a solution of monoclonal anti-T-2 toxin antibodies, a direct competitive enzyme immunoassay was performed. This assay included the incubation of the antibody-coated dipsticks in a mixture of sample and T-2 toxin–horseradish peroxidase conjugate. Afterwards, the strips were placed in a chromogen-containing substrate solution (H_2O_2–3,3′,5,5′-tetramethylbenzidine) for color reaction. The dot color intensity of toxin-positive dipsticks was visually distinguishable from that of the negative control. A portable colorimeter was used to confirm and quantify the visual observations. With coated strips, the tests could be performed in 45 min. The visual detection limit for T-2 toxin in buffer solution was 0.25 ng/ml. Artificially infected wheat samples were extracted with 80% methanol–water. A dilution of the raw extract of 1:8 was sufficient to avoid matrix effects. It was possible to make visually a clear distinction between the negative control and a wheat extract spiked with 12 ng/g.

Molds can produce highly toxic substances during their growth on foods and feeds. These toxic metabolites are designated mycotoxins. The mycotoxin dealt with in this paper is the T-2 toxin. It is one of the trichothecene mycotoxins. These are produced by members of several fungal genera, mainly by members of the genus *Fusarium*. Of over 150 known trichothecenes, only a few occur under natural conditions. T-2 toxin is one of those that have been detected in agricultural crops, especially in wheat and maize (26). It may be the cause of serious economic losses (15), and, when ingested, it can produce toxic syndromes in humans and animals in concentrations as low as micrograms or nanograms of toxin per gram of food or feed (2, 24, 26).

There is a need in the field of agricultural and veterinary diagnosis for a screening methodology for this toxin. Immunoassays of the conventional form (enzyme-linked immunosorbent assays [ELISAs]) have been successfully applied to the detection of T-2 toxin in cereals (2, 5, 7, 10, 11, 19). ELISAs are very sensitive and require little sample preparation, which makes them suitable tools for screening large numbers of samples.

The most common method for immobilization of antibodies or antigens on the microtiter plates is passive adsorption. However, it has been reported that passive adsorption of proteins on hydrophobic surfaces can produce substantial conformational and functional changes in proteins (3). To overcome this problem, surface-modified hydrophilic membrane supports that covalently bind proteins can be used. It has been proved that such membranes retain immobilized proteins in their native conformations (17).

Other drawbacks of microtiter plate immunoassays are the several hours required to complete the test, the need for sophisticated equipment and qualified personnel, and the restriction of the application to laboratories.

In recent years, diagnostic testing in the agricultural and veterinary fields has been expanding to alternate testing sites, such as farms, storehouses, and factories. To meet this market's need, the complex multistep immunoassay has to be compressed into a self-contained package. This requires novel approaches. The techniques should be simple, should use samples without manipulation or with minimal manipulations, and must provide accurate results with little or no instrumentation. Membrane substrates are valuable solid phases in immunoassays for application in the field (34). Many of these membrane-based immunoassays have been developed for a variety of applications (6, 9, 12, 14, 18, 20–22, 25, 27, 32, 33).

This paper describes the development of a dipstick for the rapid detection and semiquantitative determination of *Fusarium* T-2 toxin in wheat. A dipstick is a plastic strip to which a membrane, coated with immunoreagents, is attached and is dipped into different solutions of immunoreagents during an immunoassay. A new surface-modified hydrophilic membrane support that covalently bind proteins was used to overcome the problems associated with passive adsorption.

MATERIALS AND METHODS

Materials and reagents. T-2 toxin (T-2), Tween 20, casein sodium salt (casein), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. The membrane (Immunodyne ABC; pore size, 0.45 μm) was obtained from Pall BioSupport, Portsmouth, England. Rabbit anti-mouse immunoglobulins (no. Z 259; protein concentration, 3.2 g/liter, reacting with all mouse immunoglobulin G [IgG] subclasses, mouse IgA, and mouse IgM) were from Dako, Glostrup, Denmark. A T-2–horseradish peroxidase (HRP) conjugate was prepared by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary (2). Monoclonal antibodies against T-2 were produced and characterized by the same institute (8). Cross-reactions with T-2, acetyl-T-2, HT-2, and iso-T-2 were 100, 12.8, 3.4, and 2.5%, respectively. There was no cross-reaction with T-2 triol, T-2 tetraol, verrucarin A, verrucarol, roridin A, diacetoxyscirpenol, deoxynivalenol, or zearalenone (2, 8). The anti-T-2 monoclonal antibody was IgG1 with kappa light chains. The affinity constant was 3.2×10^{10} M^{-1}. The anti-T-2 ascites and the anti-T-2 globulin (pure immunoglobulin fraction; protein content, 1 mg/ml) were used. 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Aldrich Chemical Co. Hydrogen peroxide (30%) was purchased from Merck, Darmstadt, Germany. The substrate solution was prepared as described by

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Dürsch and Meyer (4) with little modification. This solution was made prior to the assay by mixing equal volumes of solutions A and B. Solution A (pH 5.0) contained 1.00 g of hydrogen peroxide per liter, 18.00 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 10.30 g of citric acid $\cdot H_2O$ per liter, and 0.05% Proclin 300 (Rohm and Haas, Croydon, England) as a preservative. Solution B (pH 2.4) contained 500 mg of TMB, 40 ml of dimethyl sulfoxide, 960 ml of distilled water, 10.30 g of citric acid $\cdot H_2O$, and 0.05% Proclin 300. The buffer used for dilution of anti-T-2 antibodies and T-2–HRP (assay buffer) consisted of phosphate-buffered saline (PBS) (pH 7.4) (30) containing 0.1% BSA and 0.05% Tween 20 and supplemented with 0.05% Proclin 300. Methanol and water were high-pressure liquid chromatography grade. A stock solution of T-2 (1 mg/ml) was prepared in methanol and stored at −20°C (19). A working stock solution of T-2 (100 μg/ml) was prepared in 10% methanol–PBS and stored at <5°C. Working standards of 0.25 to 25 ng/ml were prepared on the day of assay in 10% methanol–PBS. All T-2 solutions and substrate solutions were kept in the dark. The glassware and T-2 waste were decontaminated with sodium hypochlorite solution as previously described (31).

Preparation of dipsticks. The Immunodyne ABC membrane was cut into sections (1 by 1 cm). These were mounted onto plastic supports. Portions (2 μl) of undiluted rabbit anti-mouse immunoglobulins were spotted on the membrane with the CAMAG Automatic TLC Sampler (ATS3). The test strips were dried at 37°C for 30 min. The remaining protein binding sites of the membrane were blocked for 15 min by immersing the strips in PBS containing 2% casein. The dipsticks were washed with PBS–0.05% Tween 20. The next step was immersion of the anti-mouse immunoglobulin-coated test strips during 30 min in 0.5 ml of anti-T-2 globulin solution (1:10,000 in assay buffer). After being washed and dried, the coated test strips were stored in plastic vacuum-sealed bags at room temperature in the dark.

Assay procedure. A direct competitive enzyme immunoassay was used (28). The antibody-coated dipsticks were incubated for 30 min at room temperature in a mixture of 0.5 ml of T-2 standard solution or sample extract solution and 0.5 ml of T-2–HRP (1:50,000 in assay buffer). For the negative control, a solution containing 0.5 ml of 10% methanol–PBS and 0.5 ml of T-2–HRP was used. The next step was washing of the strips with PBS–0.05% Tween 20. Finally, each strip was incubated in a test tube containing 2 ml of substrate solution (H_2O_2-TMB) for 5 min. The dot color intensity of the test strip was visually compared with that of the negative control, which showed the most intense blue color because of the inverse relationship between toxin concentration and color development. The smallest toxin concentration that resulted in a color intensity clearly distinguishable from that of the negative control by several persons was considered the visual detection limit (21). Since the color was not stable for long, the reading had to be done immediately. The portable colorimeter, a Chroma Meter CR-321 (Minolta), was used to quantify the color of the dots on the T-2 dipstick. This was done by measuring the difference between the color of a white membrane (as a reference) and the dot color intensity of the test strip. This color difference was expressed as a single numerical value, ΔE^*_{ab} (13).

Extraction and treatment of wheat. Wheat was milled in a coffee grinder. A 5-g portion was mixed with 15 ml of 80% methanol–water for 1 h. The suspension was then filtered through Whatman no. 4 paper. The filtrate was diluted in PBS until a final concentration of 10% methanol was reached. This was used in the assay. Spiked samples (8 to 72 ng/g) were prepared 1 day prior to extraction. Therefore, the appropriate volume of a T-2–methanol solution (10 ng/μl) was added to 5 g of ground wheat. After 1 day, the methanol was evaporated. The spiked samples were shaken before extraction.

RESULTS

Study of the binding of antibodies to membranes. The following commercially available membranes to which proteins can be covalently immobilized were tried and compared: Immobilon affinity membrane, 0.65-μm pore size (Millipore); Immunodyne immunoaffinity membrane, 1.2-μm pore size (Pall BioSupport); and Immunodyne ABC membrane, 0.45-μm pore size (Pall BioSupport). All three membranes gave a very low background level after blocking and provided white surfaces for excellent color discrimination. The stabilities of immunoreagents immobilized on these membranes were similar when they were stored in plastic vacuum-sealed bags at room temperature and protected from light. Because these parameters were equal for the three membranes, the membrane with the highest protein binding capacity would be the best choice. For the same working dilutions of the antibodies and the T-2–HRP, the color intensity of the negative control was distinctly higher when the Immunodyne ABC membrane was used. This shows that the Immunodyne ABC membrane bound the largest amount of immunoreagents. The following experiments were done with this new membrane.

Immobilization of the anti-T-2 ascites or the anti-T-2 immunoglobulin directly on the membrane gave poor results. The colored dots were too small in area, although 2 μl was spotted. Concentrated solutions of anti-T-2 antibodies and T-2–HRP had to be used to obtain some color. Therefore, a second antibody technique was tried to overcome these problems: the membranes were coated with undiluted rabbit anti-mouse immunoglobulins (3.2 mg/ml). The spots obtained by applying 2 μl of the second antibody were more extensive. The immunoreactants could be much more diluted (anti-T-2 globulin at 1:10,000 and T-2–HRP at 1:50,000 instead of anti-T-2 globulin at 1:1 and T-2–HRP at 1:100). As the first antibody, the anti-T-2 globulin gave the best results.

First, the anti-mouse immunoglobulins were manually spotted on the membrane with an Eppendorf pipette. Because of this manual application, the reproducibility of the spot diameter was rather poor. Use of the CAMAG Automatic TLC Sampler (ATS3) was the solution to this problem. The spots were reproducible, but this procedure required more time.

After the membrane was coated with the rabbit anti-mouse immunoglobulins, the remaining covalent sites of the membrane were blocked. In this way, the undesired nonspecific binding could be suppressed. Casein has proved to be the best of all the commonly employed blocking agents for use with Pall nylon membranes (16). A 2% (wt/vol) casein-PBS solution was used. The background color became minimal.

Dipstick enzyme immunoassay to detect *Fusarium* T-2 in buffer solutions. A substrate that made low concentrations of peroxidase visible was needed. Several substrates were tried. 4-Chloro-1-naphthol (Sigma Chemical Co.) and diaminobenzidine (ImmunoPure Metal Enhanced DAB Substrate Kit; Pierce) did not give a signal at high HRP dilutions. The commonly used soluble TMB substrate gave a high color intensity with low concentrations of immunoreactants.

The working dilutions of the immunoreactants were determined in this direct competitive dipstick assay. Dilutions of 1:10,000 for the anti-T-2 globulin and 1:50,000 for the T-2–HRP resulted in an intense blue dot for the negative control. With these dilutions, it was also possible to make a visual distinction between very small changes in color intensity.

A visual detection limit of 0.25 ppb (0.25 ng of T-2 per ml of buffer solution [10% methanol–PBS]) was reached with those working dilutions. A T-2 concentration of 3 ppb suppressed the color development completely.

By using the Minolta Chroma Meter CR-321, a dose-response curve for T-2 could be established (Fig. 1). This was obtained by plotting the T-2 toxin concentration against the difference (ΔE^*_{ab}) between the color of a white membrane (as a reference) and the dot color intensity on the dipstick. The experiment was repeated 10 times on different days, covering a period of about 2 months and with dipsticks coated on different days. Means and standard deviations were calculated and are shown in Fig. 1. The coefficients of variation (CVs) were between 11 and 23%. This could be attributed to variations in the coating and incubation steps. Figure 1 shows that ΔE^*_{ab} never reached zero. This was due to background color development caused by light, air, and time. However, this background color development was minimal, and the difference between the spot and background colors remained very large. For visual semiquantitative detection, 3 ppb was chosen as a cutoff value. When there was no dot color development, the sample contained a T-2 level of 3 ppb or more. In each assay, 3 ppb (no dot color, ΔE^*_{ab} between 4.41 and 6.33 as in Fig. 1) was used as the positive control and 0 ppb (highest color intensity, ΔE^*_{ab} between 8.69 and 13.07 as in Fig. 1) was used as the negative control.

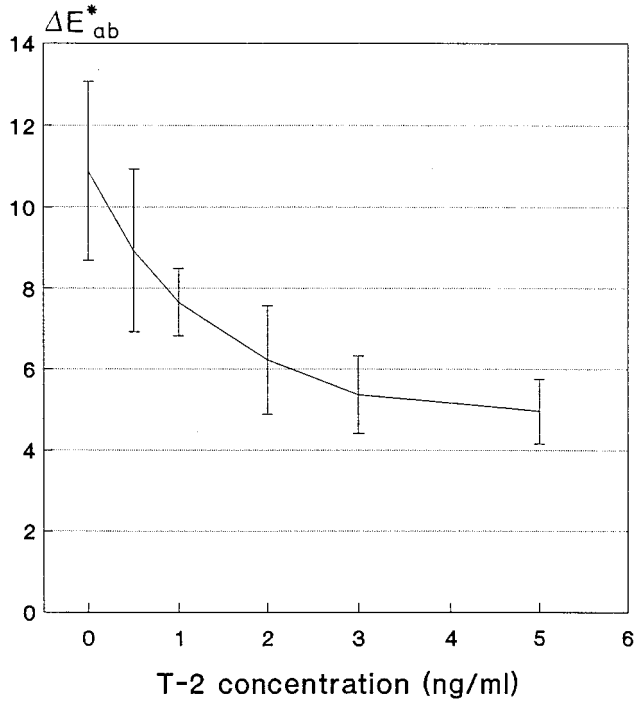

FIG. 1. Dose-response curve of the dipstick enzyme immunoassay for the detection of *Fusarium* T-2 toxin in buffer solutions. The *x* axis indicates the T-2 toxin concentration, and the *y* axis indicates the difference (ΔE^*_{ab}) between the color of a white membrane (as a reference) and the dot color intensity on the dipstick (means and standard deviations). The experiment was repeated 10 times on different days. CVs were between 11 and 23%.

It was unnecessary to use freshly prepared dipsticks for an assay. Membranes coated with the second and first antibodies could be stored for 2 weeks without any deterioration if they were kept in plastic vacuum-sealed bags at room temperature and in the dark. The room temperature over the time span of these experiments was 17 to 28°C. The color intensity of the negative control acted as a control for stability (i.e., ΔE^*_{ab} should be more than 8.69) (Fig. 1).

Dipstick enzyme immunoassay to detect *Fusarium* T-2 toxin in wheat. Different methanol-based extraction solvents (100, 80, 60, and 40% methanol in water) were tried and compared. Table 1 shows that the recovery decreased with diminishing methanol content. For 40% methanol, a high, disturbing background level made it impossible to determine the amount of T-2 recovered. When 60% methanol was used, the recovery was 50% with a CV of 49%. This must be due to the very high background color intensity. With 100% methanol, the apparent recovery was too high (133% with a CV of 23%). A methanol concentration of 80% in water gave the best results. The recovery was 95% with a CV of 18%, and the background color was minimal. This solvent was used for the extraction of T-2 from wheat.

TABLE 1. Effect of different methanol contents in extraction solvents on the recovery of T-2 from spiked wheat samples (dipstick enzyme immunoassay)

Extraction solvent (% methanol in water)	T-2 added (ng/g)	T-2 toxin found				*n*
		Mean (ng/g)	SD (ng/g)	CV (%)	Recovery (%)	
100	60	80	18	23	133	3
80	60	57	10	18	95	3
60	60	30	15	49	50	3
40	60	ND[a]				3

[a] ND, not detectable.

TABLE 2. Recovery of T-2 toxin from artificially contaminated wheat (dipstick enzyme immunoassay)

T-2 added (ng/g)	T-2 toxin found				*n*
	Mean (ng/g)	SD (ng/g)	CV (%)	Recovery (%)	
8	14	6	43	175	3
12	16	5	31	133	5
24	27	3	11	113	6
48	44	10	23	92	6
72	62	18	29	86	4

In the dipstick enzyme immunoassay, however, it was not possible to use such a high methanol concentration. The use of greater than 10% methanol resulted in a significant color reduction. Before the assay, the raw methanolic extract was diluted in PBS until a final methanol content of 10% was reached.

Further cleanup was not necessary. The dilution of the raw extract of 1:8 was sufficient to avoid matrix interference. A blank wheat extract resulted in a color intensity similar to that of the negative control. Sometimes it gave higher ΔE^*_{ab} values than the negative control. Blank extracts were always clearly considered negative compared with the negative control. Recoveries of T-2 from artificially contaminated wheat were determined (Table 2). When very small amounts of T-2 (8 and 12 ng/g) were spiked, the apparent recoveries were too high (175 and 133%, respectively). The CVs were more than 30%. The recoveries at 24, 48, and 72 ng/g were 113, 92, and 86%, respectively, with CVs of between 11 and 29%. False-negative results were never obtained. It was possible to make visually a clear distinction between the negative control and a wheat extract spiked with 12 ng/g.

DISCUSSION

Study of the binding of antibodies to membranes. Membrane substrates offer several technical advantages over conventional solid phases (34). Membranes have a higher protein binding capacity due to their porosity and consequential large surface area. With conventional solid phases the kinetics of reactions between immobilized and soluble reactants are limited by diffusion of the soluble, mobile reactants to the insoluble surface components (29). Because of their flowthrough capability, membranes permit the liquid component to be drawn actively through the pores. This brings the soluble reactants rapidly into close contact with the reactive sites on the surface (35). As a consequence, the assay time will be significantly reduced. However, not all problems associated with conventional solid phases (e.g., nonspecific protein binding) can be solved by membranes. Proteins bind to many membranes through a hydrophobic, electrostatic, or ionic interaction. This binding is indiscriminate and reversible. Schneider et al. (21) reported that the sensitivity and reproducibility of a solid support immunoassay for a specific antigen is limited by the ability of the antibodies to remain bound to the matrix under the assay conditions. Furthermore, passive adsorption of proteins on hydrophobic surfaces is an inactivation process. Butler et al. (3) reported that most antibodies were denatured by passive adsorption. Surface-modified hydrophilic mem-

brane supports that covalently bind proteins offer a major solution to these problems. Proteins bound to such membranes are retained in their native conformation (17). The commercially available membranes compared in this study provided covalent binding of the antibodies. The Immunodyne ABC membrane was the best because of the greater protein binding capacity and the smaller pore size of that new membrane. It is a nylon membrane which has been surface modified with reactive groups to covalently bind proteins and other compounds containing amino groups.

The poor results obtained when the anti-T-2 ascites was immobilized directly on the membrane could be caused by the nonspecific binding of impurities. The protein content of the original anti-T-2 globulin solution was 1 mg/ml. A protein concentration of 1 to 10 mg/ml and a volume of between 1 and 3 μl are needed for coating of an Immunodyne membrane (16). Thus, the anti-T-2 globulin would have to be used undiluted, which would be too expensive and which would result in a low sensitivity of the assay. Therefore, a second antibody technique was used, with improved results. Similar observations were made by other authors. Schneider and Hammock (23) and Giersch (6) reported that the use of anti-mouse immunoglobulin coating for trapping monoclonal antibodies provided the best sensitivity and reproducibility in their immunoassays. This was due to the low concentrations of antibodies and extremely high dilutions of enzyme tracers which could be used. Furthermore, Butler et al. (3) proved that antibodies immobilized by using an antiglobulin retained full activity.

Dipstick enzyme immunoassay to detect *Fusarium* T-2 in buffer solutions. The first step in developing a dipstick enzyme immunoassay is the determination of the optimal antibody and conjugate dilutions. For a microtiter plate ELISA this can very easily be done by checkerboard titration. For a dipstick assay this is not such an easy task, and it takes a lot of time. The purpose of a dipstick is to allow visual evaluation. It is more difficult to see differences between two dark colors than between two pale colors or between no color and a pale color. The color intensities of the spots must be high enough to be seen and low enough to enable observation of differences in color intensity. When the working dilutions are high, the assay is more sensitive. Singh and Jang (25) reported that the sensitivity of a membrane-based assay could be controlled to a desired level by adjusting the amount of immobilized antibody on the solid support. Decreasing the immobilized antibody concentration increased the sensitivity of the assay within a certain limit. However, when the working dilutions are too high, the color will be too pale. The optimal dilutions for the anti-T-2 globulin and the T-2–HRP had to be determined by trial and error.

With those working dilutions, a visual detection limit of 0.25 ppb was reached, and 3 ppb suppressed the color development completely. Compared with other test strips, the developed dipstick is very sensitive. Schneider et al. (21, 22) reported a visual detection limit of 1 ng/ml for the test strip with T-2 in buffer solution. A T-2 concentration of 5 ng/ml suppressed the color development completely. Compared with the conventional microtiter plate ELISA, this dipstick is very sensitive as well. Barna-Vetro et al. (2) reported a detection limit for T-2 in buffer solution of 0.43 ng/ml, and Lacey et al. (11) reported 10 ng/ml as the minimal concentration of T-2 that could be determined.

Figure 1 is not linear. However, the dipstick enzyme immunoassay was not developed for quantitative determinations. Between 0 and 3 ppb there is an important decrease in dot color intensity. From 3 ppb there is a plateau with only very little background color. A similar standard curve for the detection of mycotoxins by a competitive line immunoblot assay with monoclonal antibodies was obtained by Abouzied and Pestka (1).

Dipstick enzyme immunoassay to detect *Fusarium* T-2 toxin in wheat. Extraction and sample pretreatment were kept as simple as possible. Methanol in combination with water is often used for the extraction of T-2 from cereals (5, 10, 26). Aqueous solvents readily penetrate hydrophilic tissues and enhance toxin extraction. The correct concentration of water further enhances the partition of mycotoxins in the solvent (26). In this assay wheat was extracted with 80% methanol–water. The raw extract was diluted until a final methanol content of 10% was reached. The use of more than 10% methanol resulted in a significant color reduction. Barna-Vetro et al. (2) reported similar observations: in the range of 10 to 100%, ethanol and acetonitrile inhibited the binding of the toxin-peroxidase conjugate to the solid-phase antibody. This effect could be avoided by decreasing the acetonitrile or ethanol concentration to less than 10%. A further cleanup of the extract was not necessary.

Blank wheat extracts sometimes gave higher color intensities than the negative control. Laamanen and Veijalainen (10) reported similar observations. The foods and feeds which gave stronger color reactions were most often freshly milled grain. Those authors explained these elevated reactions by the activity of endogenous peroxidases present in extracts of plant origin. Careful washing between the ELISA steps did not completely remove these substances from their microtiter plates. Because of the high sensitivity of our T-2 dipstick enzyme immunoassay, that interference did not give false-negative results.

When the wheat was spiked with 12 ng/g, the dot color intensity of the dipstick was clearly distinguishable, by seven persons, from that of the negative control. Compared with other reports, this is a very good result. Schneider et al. (21) reported for their test strip a visual detection limit of 20 ng/g for T-2 in wheat.

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