Development of Immunoassays for Tyramine and Tryptamine Toxins of *Phalaris aquatica* L.

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The leaves of the perennial pasture grass *Phalaris aquatica* L. (phalaris) contain two groups of known toxins, indole alkaloids, primarily dimethyltryptamines and *N*-methyltyramines, which cause illnesses in grazing animals, especially sheep. Using amino-reactive and phenolic hydroxyl-reactive homobifunctional reagents, simple methods were devised for coupling toxins representative of those in phalaris to carrier proteins and enzymes for ELISA development. ELISAs were produced for both groups of toxins. Dimethyltryptamines were most sensitively detected [lower limit of detection (LLD) of 1 μg/L for bufotenine] using rabbit anti-bufotenine antibodies, coupled to ovalbumin using divinyl sulfone, with detection using a peroxidase conjugate prepared using the same hapten coupled with 1,4-butanediol diglycidyl ether. The assay cross-reacted with other toxins of the same class (*N*,*N*-dimethyltryptamine and *N*,*N*-dimethyl-5-methoxytryptamine) but not with the structurally related amino acids histidine and tryptophan. The most sensitive *N*-methyltyramine assay (LLD of 1 μg/mL for *N*-methyltyramine) utilized antisera to tyramine with *N*-methyltyramine coupled to peroxidase. Significant cross-reaction was seen with the low-grade toxin hordenine, but detection of tyramine was poorer, whereas the amino acid tyrosine was not detected. These assays could be applied to the analysis of simple extracts of *Phalaris* leaves with minimal interference. A good correspondence was observed between toxin levels by ELISA and estimates from a more tedious thin-layer chromatography method. The method has now been incorporated in a *Phalaris* breeding program.

Keywords: *Immunoassay; Phalaris; grass; toxin; dimethyltryptamine; N-methyltyramine*

INTRODUCTION

Phalaris species provide valuable pasture growth for grazing in temperate regions of southeastern Australia, Argentina, California, Europe, and northern and southern Africa, where the productivity and survival of other perennial grasses is limited by dry summers. However, a significant shortcoming is that these species produce a series of alkaloid toxins, especially in growth following drought (East and Higgins, 1988; Anderton et al., 1994; Cheeke, 1995). Toxicity and deaths have been reported in Australia (Bourke et al., 1990; Bourke and Carrigan, 1992), South Africa (Van Halderen et al., 1990), Argentina (Odriozola et al., 1991), and the United States (Lean et al., 1989). The relative responsibility of different alkaloids (and other, as yet, uncharacterized toxins) for morbidity and mortality in sheep is the subject of some debate (Culvenor, 1987; Bourke et al., 1990; Edgar, 1994). Selection for low toxin levels remains an important focus of the breeding program in Australia (Oram and Culvenor, 1994). The established *Phalaris* alkaloid toxins fall into two classes: the indole alkaloids (which include dimethyltryptamine, bufotenine, 5-methoxy-*N*,*N*-dimethyltryptamine, gramine, and the cyclization product 2-methyl-1,2,3,4-tetrahydro-β-carboline) and the tyramines (*N*-methyltyramine and hordenine) (Figure 1). The indole alkaloids have been associated with irreversible "*Phalaris* staggers" in sheep, whereas consumption of pasture containing *N*-methyltyramine, especially of new growth following a late autumn moisture stress, has been associated with sudden death of sheep (Anderton et al., 1994).

Earlier studies have shown that consistent genotypic variation in toxin levels makes breeding for low toxin levels an achievable aim (Oram and Williams, 1967; Oram and Schroeder, 1987, 1992). One limitation in selection for low toxin lines in breeding is that the thin-layer chromatography method for screening grass samples for these toxins (Anderton et al., 1994, 1998) was rather labor-intensive and limited in sample throughput, largely because the sample preparation and cleanup involved six steps. Gas chromatographic analyses for some of the *Phalaris* alkaloids have also been described (Audette et al., 1969), but these are also labor-intensive. We thus investigated whether the development of an immunoassay for the *N*-methyltyramine and *N*,*N*-dimethyltryptamine toxins was a viable alternative, but we did not investigate development of an ELISA assay for the less common β-carboline toxins.

Although the target molecules are closely related to protein-forming amino acids, immunoassays have successfully been developed for physiologically active tryptamines, including radioimmunoassays detecting either 5-hydroxytryptamine (Engbaek and Voldby, 1982) or three of the dimethyltryptamines (*N*,*N*-dimethyl-

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10.1021/jf990452z CCC: $19.00 Published 2000 by the American Chemical Society
Published on Web 12/04/1999

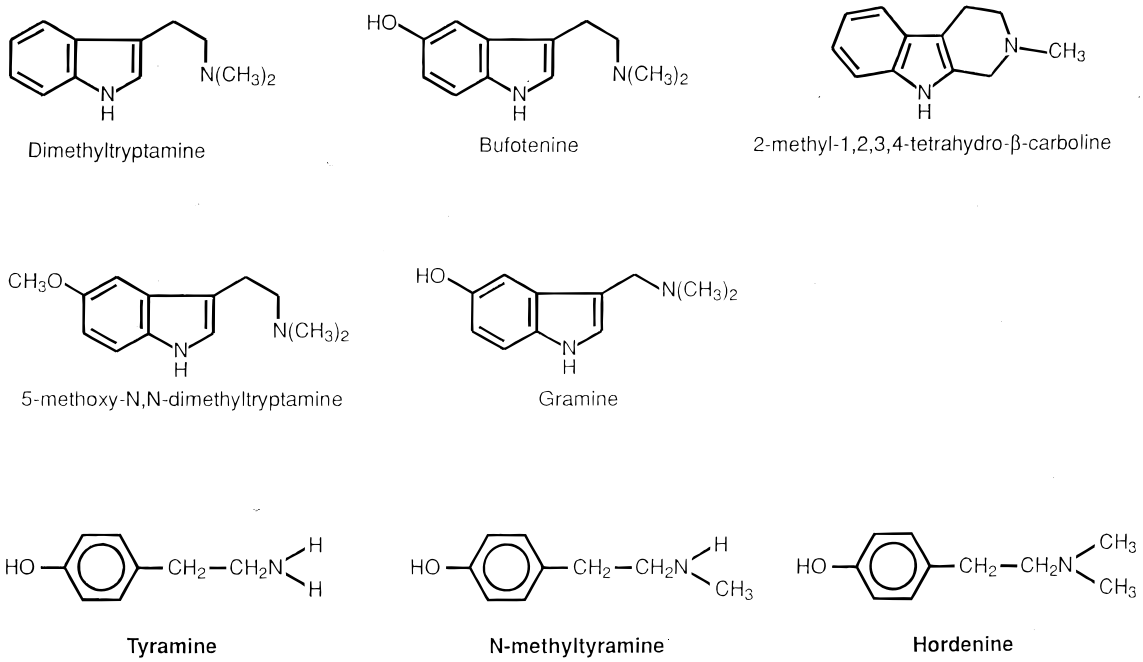

Figure 1. Structures of the alkaloids in major toxin groups from *Phalaris* species: dimethyltryptamines (dimethyltryptamine, BUF, 5-methoxy-*N*,*N*-dimethyltryptamine, gramine), tyramines (TYR, NMT, hordenine), and β-carbolines (2-methyl-1,2,3,4-tetrahydro-β-carboline).

tryptamine, bufotenine, and 5-methoxy-*N*,*N*-dimethyltryptamine) (Riceberg and van Vunakis, 1978). However, these assays were not adapted into the simpler ELISA format, and, as in the case of an ELISA for ergotamine (Shelby and Kelley, 1990), they were applied only to urine, plasma, and blood, rather than plant materials. In this paper, we describe the development of ELISAs for both families of toxins, using simple protein coupling chemistry involving homobifunctional reagents. The assay has been successfully adapted to the analysis of direct extracts of grass leaves and has been integrated into the selection procedure of the CSIRO *Phalaris* breeding program.

MATERIALS AND METHODS

Materials. Bufotenine (BUF), tyramine (TYR), *N*-methyltyramine (NMT), and *N*,*N*-dimethyltryptamine (purchased from Aldrich, Milwaukee, WI); L-histidine, L-tryptophan, and 5-hydroxytryptamine (purchased from Sigma, St. Louis, MO); and 5-hydroxy-*N*-ω-methyltryptamine (K&K Laboratories, Plain View, NY) and hordenine sulfate (Fluka, Buchs, Switzerland) were prepared in distilled water. *N*,*N*-Dimethyl-5-methoxytryptamine and gramine (Aldrich), *N*-ω-methyltryptamine (Fluka), and l-tyrosine (Sigma) were provided as free bases and thus were dissolved at 1 mg/mL in 50 mM HCl before analysis in ELISA.

Extraction of *Phalaris* Leaves and Toxin Analysis by Thin-Layer Chromatography (TLC). The expanding and youngest fully expanded leaves were plucked from several (>4) tillers on the northern (sun-facing) side of vegetative *Phalaris* plants in mid-late June (i.e., Australian winter) and chopped into 1 cm lengths. A 4.0 g subsample was taken and immersed in a vial containing 45 mL of 0.1 M HCl. The samples were kept refrigerated and shaken daily for 4 days, after which time the leachate was filtered and stored at −16 °C. These extracts were either directly used for ELISA or processed for TLC. For TLC, the alkaloids were adsorbed onto a strong cation exchange solid-phase extraction cartridge (Alltech, Deerfield, IL) and eluted with 6 M ammonia in ethanol (Anderton et al., 1998). The alkaloids were separated on silica gel 60 F-254 TLC plates (Alltech catalog no. 7467) and visualized according to the method of Anderton et al. (1994). The color intensity of each spot was rated on a 0−6 scale on which the standards were rated 4 (Oram and Edlington, 1996). Standards equivalent to 3.5 mg/kg of fresh weight of leaf tissue for dimethyltryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, BUF, gramine, β-carboline, NMT, TYR, and hordenine were run on each plate.

Preparation of Toxin−Protein Conjugates. Several of the target alkaloid toxins (or their analogues) are available commercially and have primary or secondary amino groups and/or phenolic hydroxyls that can be use for coupling, through a suitable spacer arm, to carrier proteins. (Note: because of potentially hallucinogenic properties of compounds such as BUF, a government permit may be required to purchase and store the compounds in some countries.) Each of the couplings was also performed to ovalbumin (OA) for antibody production and to keyhole limpet hemocyanin (KLH), to enable these conjugates to be used for initial antibody assessment by indirect ELISA, and to horseradish peroxidase (HRP) for use in the final competitive ELISA format.

Three dimethyltryptamine conjugates were prepared:

1. BUF (5-hydroxydimethyltryptamine) was coupled to protein through the phenolic hydroxyl group to ϵ-amino groups on protein lysine residues using divinyl sulfone (DVS) (Houen and Jensen, 1995) (conjugates termed BUF−DVS−). This was carried out by first reacting the carrier protein (10 mg of OA or KLH or 5 mg of HRP) in 3 mL of 0.1 M Na_2CO_3 buffer, pH 10, with 5 μmol of DVS overnight at room temperature. The unreacted DVS was removed by gel filtration, and then 5 mg of BUF was immediately added to the activated protein and mixed overnight at room temperature. Free BUF was then removed by dialysis against PBS. This chemical approach produces a five-atom spacer arm with a sulfone group.

2. BUF was also coupled through the phenolic hydroxyl group to protein using 1,4-butanediol diglycidyl ether (BDE; Lommen et al., 1995) (conjugate termed BUF−BDE−). The overall chemical strategy is similar to that for conjugate 1, except that the spacer arm is somewhat longer. Fifty micromoles of BDE (10 μL) was added to 20 mg of OA or 10 mg of KLH or HRP in 1 mL of 50 mM sodium carbonate, pH 10.6, and the solution mixed overnight at room temperature. The solution was then desalted on a D-6 column (Bio-Rad, Hercules, CA), and 10 mg of BUF was added to the BDE-derivatized protein. After mixing overnight at room temperature, the mixture was extensively dialyzed against 50 mM sodium phosphate−0.9% NaCl, pH 7.2 (PBS).

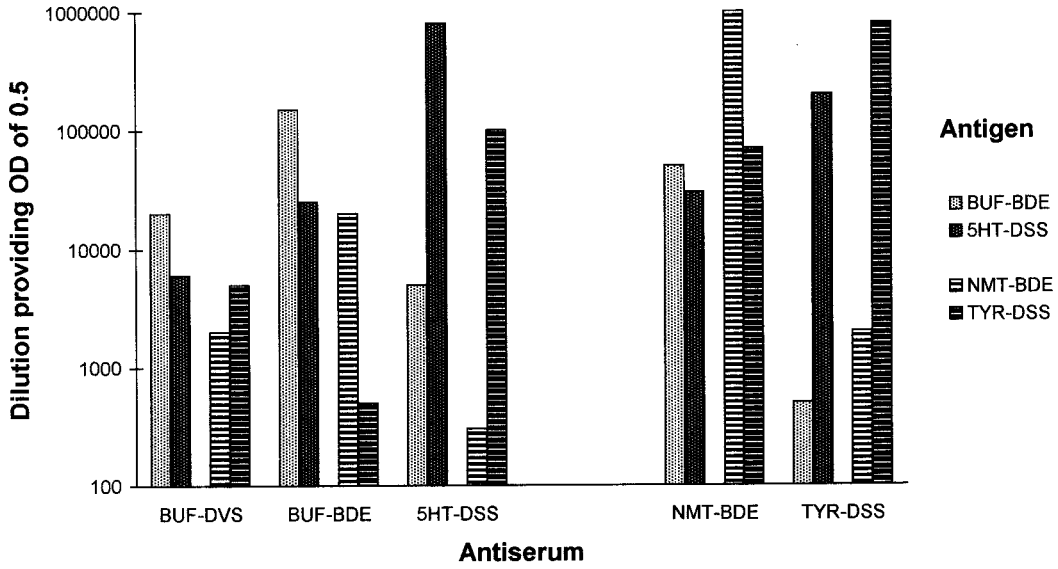

Figure 2. Detection of toxin−protein conjugates in indirect ELISA. Antisera to OA−toxin conjugates were titrated against each KLH−toxin conjugate. Data shown are dilutions of sera producing an OD of 0.5 for KLH−toxin conjugates immobilized at 0.5 μg/well and are geometric means of two determinations.

3. 5-Hydroxytryptamine was coupled through its primary amine group to amino groups on protein lysine residues using disuccinimidylsuberimidate (DSS; Paek et al., 1993) (conjugate 5HT−DSS−). The 5-HT solution (10 mg in 1 mL) was added to DSS (20 mg in 1 mL in dry DMSO), providing a 2 M excess of cross-linker. The mixture was stirred for 30 min at room temperature and then added to 10 mg of OA or KLH or 5 mg of HRP protein in 2 mL of 10 mM sodium phosphate buffer, pH 7, and a 20 M excess of hapten was used. After incubating for 2 h at room temperature, the mixture was dialyzed against PBS. This chemical approach was chosen to couple the tryptamine structure through the opposite end of the molecule to conjugates 1 and 2, thus presenting the molecule differently to the immune system for antibody production. DSS was chosen because, unlike the other cross-linkers, it will specifically react with the amine group of 5-HT rather than the phenol.

Two NMT conjugates were prepared:

4. TYR was coupled to each protein using DSS, employing the method described as for conjugate 3. This produces a conjugate that resembles NMT as it is a secondary amine, coupled through the amino group, and retaining a free phenol.

5. NMT was coupled through the phenolic group to each protein using BDE, as for conjugate 2. This produces a conjugate in which the NMT is coupled to protein in the opposite configuration to conjugate 4, leaving the *N*-methyl group exposed to the immune system.

Immunizations and Assessment of Serum Titers by Indirect ELISA. One milligram of each of the five conjugates with OA in 50 mM PBS, pH 7.2, was emulsified with an equal volume of Freund's complete adjuvant and immunized subcutaneously and intramuscularly in divided doses into different New Zealand white rabbits. Two and four weeks later, booster doses of 0.5 mg of conjugate, emulsified in Freund's incomplete adjuvant, were administered. Nine days later, blood was collected from the lateral ear vein, and sera were prepared after coagulation and centrifugation. The sera were titrated against each of the KLH−toxin protein conjugates by indirect ELISA, using 5 and 50 ng/well of conjugate. Stocks of protein conjugates were diluted in 50 mM sodium carbonate buffer, pH 9.6, and 100 μL/well coated overnight onto Maxisorp C (Nunc, Roskilde, Denmark) plates at room temperature. Plates were then washed three times in PBS−0.05% Tween 20 (PBS-T). Blocking solution [150 μL/well of 1 % bovine serum albumin (BSA) in PBS)] was added to each well and incubated for 1 h at room temperature. Individual sera were then serially diluted 4-fold in blocking solution, starting at a 1/500 dilution. After gentle mixing, the plates were incubated at room temperature for 1 h and then washed three times in PBS-T, before peroxidase-labeled swine anti-rabbit immunoglobuins (Dako, Carpinteria, CA), diluted 1/500 in BSA−PBS-T, were added. The plates were then washed five times in PBS-T, and hydrogen peroxide substrate/3,3′,5,5′-tetramethylbenzidine chromogen was added to each well (Hill et al., 1991) and incubated for 30 min at room temperature. Color development was stopped with 50 μL/well of 2.5 N H_2SO_4, and absorbances were read at 450 nm. The IgG fraction of each of the antisera was purified using affinity chromatography (Akerstrom et al., 1985) on recombinant protein G−Sepharose (Pharmacia, Uppsala, Sweden) for use in a quantitative immobilized-antibody ELISA for the toxins.

Development of ELISAs for *Phalaris* Alkaloids. Antisera that provided high titers in indirect ELISA were adapted to an immobilized antibody competition ELISA format. Plates were coated with 1 μg/well purified antibody in 50 mM sodium carbonate buffer, pH 9.6, and washed three times with PBS-T, and then blocking solution was added and the plates were incubated 1 h at room temperature. *Phalaris* toxin standards were prepared at 1000 ppm in distilled water if salts or in 50 mM HCl if free bases. Standards were then serially diluted in distilled water. Either diluted toxin standard or *Phalaris* grass extract, diluted 1 in 20 in water (100 μL), was added to each well, immediately followed by 100 μL of toxin−enzyme conjugate, diluted in 0.5% (w/v) fish skin gelatin (Sigma) in PBS. After gentle mixing, the plates were incubated at room temperature for 1 h. The plates were then washed five times in PBS-T, and substrate−chromogen mixture was added to each well and incubated for 30 min at room temperature. Color development was stopped with the addition of 50 μL/well of 2.5 N H_2SO_4, and absorbances read at 450 nm.

RESULTS AND DISCUSSION

Antibody Response to Toxin Conjugates. The sera were titrated against each of the KLH−toxin protein conjugates by indirect ELISA, using 5 and 0.5 μg/well of conjugate. Figure 2 shows the serum dilution providing an absorbance of 0.5 in the indirect ELISA with 0.5 μg/well antigen; several of the sera produced strong responses at over a 1 in 1 million dilution. Each of the five antisera, using the three coupling chemistries, produced antibodies that bound in a hapten-specific manner. This suggests that the coupling was successful and had proceeded as expected. For unknown reasons, the BUF−DSS−KLH conjugate nonspecifically

bound the swine anti-rabbit peroxidase second antibody conjugate used in the assay, producing extremely high background levels in each of the ELISAs, so an assessment of binding to this conjugate in indirect ELISA was not possible. Although the antisera to the dimethyltryptamines detected the TYR−KLH derivatives and vice versa, as expected, higher titers were observed with similar antigens, that is, dimethyltryptamine−OA antisera with dimethyltryptamine−KLH conjugates. Similarly, antisera to *N*-methyltyramine−OA and TYR−OA conjugates detected the corresponding KLH−TYR derivatives better. High titers to conjugates that used different spacer arm chemistry (i.e., had only the alkaloid part of the molecule in common or available for cross-reaction, e.g., antisera to BUF−BDE−OA with 5-HT−DSS−KLH conjugates) suggested that the antibodies should be able to detect free toxin.

Specificity and Sensitivity of Inhibition by Free Toxins. Each of the antibodies was purified and immobilized at 1 μg/well and assessed with each of four peroxidase conjugates (BUF−BDE−HRP, 5-HT−DSS−HRP, TYR−DSS−HRP, and NMT−BDE−HRP). The peroxidase conjugates prepared using DVS were not enzymically active, probably due to reaction of DVS with amino acid residues that were critical for enzymic activity. Those combinations that provided high ODs at low conjugate concentrations were then assessed for sensitivity and specificity for detection of the different toxins using competition ELISA. In addition, related amino acids and secondary metabolites were evaluated as they could potentially produce spurious results if detected in the assays. The antibody−peroxidase conjugate combinations that provided the most sensitive detection of the dimethyltryptamines (Figure 3A) were as follows:

1. Anti-BUF−DVS−OA with BUF−BDE−HRP, 800 ng/mL, provided the most sensitive format with a 50% B_0 (50% of maximal binding) for BUF close to 10 μg/L and an 85% B_0 (limit of detection) of ∼1 μg/L.

2. Anti-BUF−BDE−OA with BUF−BDE−HRP, 80 ng/mL, had a higher limit of detection, with a 50% B_0 (50% maximal binding) of 100 μg/L and an 85% B_0 (limit of detection) of 20 μg/L (Figure 3B). Both assays had a high cross-reaction with *N*,*N*-dimethyltryptamine and *N*,*N*-dimethyl-5-methoxytryptamine and a significant cross-reaction with other dimethylated tryptamine toxins. This is desirable as our intention was to develop the assay as a class-specific test for toxins of the indole alkaloid family. Cross-reaction (Table 1) with the amino acids histidine and tryptophan, which could interfere in the determinations, was minimal.

The combinations providing most sensitive detection of NMT (Figure 3B) were as follows:

1. Anti-TYR−DSS−OA with NMT−BDE−HRP, 650 ng/mL, provided the most sensitive format, with a 50% B_0 value of 50 μg/L and an 85% B_0 (limit of detection) of 2 μg/L.

2. Anti-NMT−BDE−OA with NMT−BDE−HRP, 25 ng/mL, had a 50% B_0 of 500 μg/L and an 85% B_0 of ∼50 μg/L. Significant cross-reaction (Table 1) was seen with hordenine (as desired), detection of TYR was poorer, and the amino acid tyrosine was fortunately not detected in either assay.

The sensitivity data provided for the assays refer to detection of standards in buffer. Studies on the levels of indole alkaloids in *Phalaris* have shown ranges from 1 to 87 mg/100 g of dry matter (Oram and Williams, 1967) and from 0.4 to 0.5 mg/100 g of fresh weight for cv. Sirolan, 2.9 mg/100 g for cv. Sirosa, and 9.2 mg/100 g for the slow winter-growing group (Oram and Schroeder, 1992). Thus, even the low-toxin lines such as cv. Sirolan would have >300 μg/L in the undiluted HCl extract if the compound were quantitatively extracted using the standard procedure. The assays have limits of detection ranging from 1 to 20 μg/L in buffer, providing the sensitivity required for trace analysis.

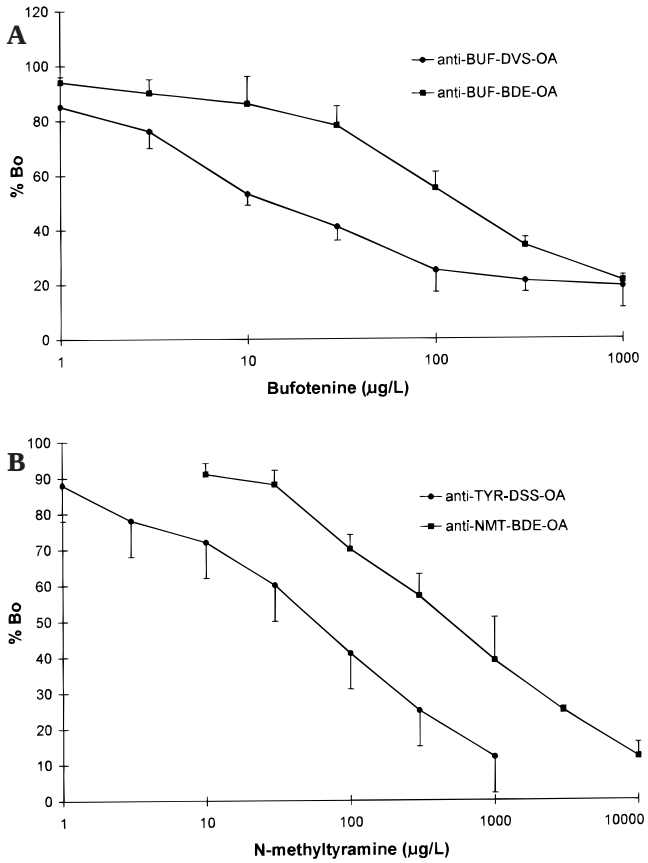

Figure 3. Standard curves for (A) *N*,*N*′-dimethyltryptamine assay (BUF) with antibodies to BUF−BDE−OA and BUF−DVS−OA and (B) NMT with antibodies to TYR−DSS−OA and NMT−BDE−OA. Each curve is the mean ± standard deviations of three to four assays.

Application to *Phalaris* Samples. Usually, the initial step in applying a newly developed immunoassay to a target matrix is to compare inhibition curves produced by standards in buffer with standards in an extract of a toxin-free sample of the matrix (Skerritt and Rani, 1996). Because no available *Phalaris* samples were totally free of toxins, we had to perform this process using a very low toxin sample (cv. Holdfast). Standard curves of dimethyltryptamine and NMT were prepared in a 0.1 M HCl extract diluted 1/10, 1/20, 1/50, and 1/100 in BSA−PBS-T. Even though the diluent efficiently neutralized the pH of the extract at a 1/10 dilution, the maximal OD and inhibition sensitivity were significantly decreased for both assays. It was not possible to determine whether this result was due to trace levels of toxin or to interference from other grass leaf components. At 1/20 dilution, the curves for standards in buffer and standards in *Phalaris* extract were superimposable, and several samples that did not have detectable levels of either toxin family by TLC also did not inhibit color development in the ELISA. Use of higher dilutions such as 1/50 and 1/100 meant that some

Table 1. Cross-Reaction of Selected ELISAs for Dimethyltryptamines and *N*-Methyltryptamines[a]

	antiserum to:	dimethyltryptamines				*N*-methyltyramines			
	HRP conjugate:	BUF−DVS−OA BUF−BDE−HRP		BUF−BDE−OA BUF−BDE−HRP		TYR−DSS−OA NMT−BDE		NMT−BDE−OA NMT−BDE	
compound		IC_{50}	IC_{10}	IC_{50}	IC_{10}	IC_{50}	IC_{10}	IC_{50}	IC_{10}
N,*N*-dimethyltryptamine		6	0.3	100	6	>1000	250	>1000	>1000
5-hydroxy-*N*,*N*-dimethyltryptamine		10	1	100	10	>1000	>1000	>1000	>1000
N-ω-methyltryptamine		1000	25	>1000	50	>1000	1000	>1000	>1000
5-hydroxy-*N*-ω-methyltryptamine		400	20	>1000	200	>1000	>1000	>1000	>1000
5-methoxy-*N*,*N*-dimethyltryptamine		1	0.05	80	6	>1000	>1000	>1000	>1000
L-tryptophan		>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000
5-hydroxytryptamine		>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000
L-histidine		>1000	200	>1000	>1000	>1000	>1000	>1000	>1000
N-methyltyramine		>1000	>1000	>1000	>1000	50	1	500	50
N,*N*-dimethyltyramine (hordenine)		1000	20	>1000	>1000	30	0.6	250	10
tyramine		>1000	>1000	>1000	>1000	>1000	170	>1000	230
L-tyrosine		>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000

[a] Data shown are concentrations in ng/mL. IC_{10} = limit of detection.

samples which had detectable levels of either or both toxins by TLC were not detectable in the ELISA. The assay with the more sensitive BUF−DVS−OA antibody was inconvenient to use as it required somewhat higher dilutions for optimal discrimination of different levels of toxin. The more sensitive TYR assay, utilizing antibody to TYR−DSS−OA, was selected for TYR analysis, whereas the antiserum to BUF−BDE−OA was selected for dimethyltryptamine analysis. This combination enabled both toxin groups to be screened using a 1/20 dilution of the grass extract.

Twenty-six samples were tested by TLC, and a comparison was made with the ELISA results. TLC plates were separately scored for four indole alkaloids (dimethyltryptamine, 5-methoxyl-*N*,*N*-dimethyltryptamine, BUF, and gramine) and three tyramines (TYR, hordenine, and NMT). Although at best it represents an approximation, a cumulative score was developed for samples of each toxin family by adding the TLC scores (see Materials and Methods) for each of the three or four toxins developed (the higher the score, the higher the toxin level). This TLC score was compared with the ELISA data, which were classified into deciles based on percent B_0. For example, a sample with between 21 and 30% B_0 for a particular toxin family was rated as 3, between 91 and 100% B_0 as 10, etc. Thus, the higher the toxin level, the lower the ELISA score.

There was an excellent correlation between TLC levels of the tyramines (Figure 4A) and ELISA scores. Samples with high levels of tyramines/*N*-methyltyramines by TLC (e.g., 11 and 12) had high levels in the ELISA (score of 3); those with intermediate TLC scores had intermediate ELISA scores, and those with low or undetectable levels of toxin by TLC had low or undetectable levels of tyramines by ELISA. The relationship between TLC and ELISA data for indole alkaloids (Figure 4B) was more complex. Samples that were high in indole alkaloids by TLC were also high by ELISA, and several of the samples that were low by TLC were also low by ELISA. However, about one-third of the samples that had low or intermediate total indole alkaloid levels by TLC were strongly positive in ELISA. Thus, use of the ELISA to cull samples based on high apparent indole alkaloid toxin levels (ELISA score of 2 or 3) would cull out all of the samples with high indole alkaloid levels by TLC but also cull out some with undetectable levels by TLC. Because the alkaloid toxins of *Phalaris* are complex and poorly characterized, it is possible that this discrepancy is due to other indole alkaloids which cross-react in the ELISA. Given that

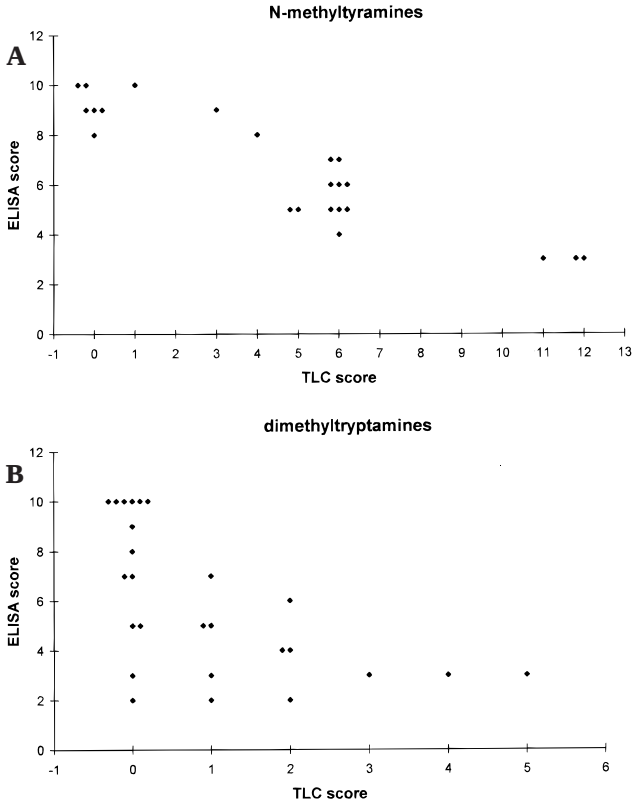

Figure 4. Comparison of TLC scores and ELISA results (%B_0 deciles, see text) for (A) *N*-methyltyramines and (B) dimethyltryptamines for extracts of 27 *Phalaris* samples.

the aim of many selection methods in plant breeding is to cull out undesirable lines, the fact that the assay reliably identified all samples that were high in toxins was more critical to its use. This assay has now been used to screen >500 single plant selections from the 1997 and 1998 *Phalaris* breeding populations at our institute.

Conclusion. We have used three homobifunctional reagents to activate protein for conjugation of dimethyltryptamine and TYR haptens to protein for antibody production and use in an ELISA reporter conjugate. The earlier radioimmunoassays developed for 5-HT (Engbaek and Voldby, 1982) and dimethyltryptamines (Riceberg and van Vunakis, 1978) were also produced by reacting the hapten with activated protein, but in this case the protein was diazotized *p*-aminophenylalanine−protein conjugate. Given that spacer arm het-

erology is often important for optimizing ELISA sensitivity (Gee et al., 1995), the DVS, DSS, and BDE reagents provide spacer arms that are 5, 10, and 10 atoms long, respectively, and can be reacted with hapten amino groups and in some cases phenols. These reagents have found some use for other hapten−protein conjugates (Paek et al., 1993; Houen and Jensen, 1995; Lommen et al., 1995) but should be applicable for ELISA development for other alkaloids and drugs, especially because specialized organic synthesis experience and facilities are not required. A disadvantage is that it is difficult to monitor the success of derivatization and coupling, unless radiolabeled haptens are used. We have developed highly sensitive ELISA assays for analysis of the tyramine and indole alkaloid classes of toxins that are found in *Phalaris* leaves. Along with other selection characteristics in *Phalaris* breeding, including winter activity, level of summer dormancy, high seed retention to facilitate seed harvesting, and tolerance to acid soils, the assays can be used to select for low toxin levels, and potentially, to be developed into an on-farm screen for pastures that may be potentially toxic due to environmental conditions.

ABBREVIATIONS USED

BDE, 1,4-butane dioldiglycidyl ether; BSA, bovine serum albumin; BUF, bufotenine; DSS, disuccinylsuberimidate; DVS, divinyl sulfone; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; 5-HT, 5-hydroxytryptamine; KLH, keyhole limpet hemocyanin; LLD, lower limit of detection; NMT, *N*-methyltyramine; OA, ovalbumin; PBS, phosphate-buffered saline; TYR, tyramine.

ACKNOWLEDGMENT

We are grateful to Rex Oram for advice and interesting us in the issue of toxin quantification, to Steve Colegate (CSIRO Animal Health, Geelong, Australia) for advice, and to Peggy Kooij for assistance with some of the immunoassays.

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Received for review May 3, 1999. Revised manuscript received August 25, 1999. Accepted September 28, 1999.

JF990452Z