

Enzyme Immunoassay for the Macrocyclic Trichothecene Roridin A: Production, Properties, and Use of Rabbit Antibodies

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Antisera against roridin A were prepared by using a roridin A-hemisuccinate derivative coupled to human serum albumin as the immunogen. Antibodies could be detected in the sera of the immunized rabbits as early as 4 weeks after the initial exposure. After one booster injection at week 14, high antibody titers were measured over a period of 21 weeks. The specificity and sensitivity of the antibodies were tested by using roridin A-hemisuccinate coupled to horseradish peroxidase as an enzyme-linked toxin in a competitive assay with a double-antibody solid phase. The assay was most specific for the tested macrocyclic trichothecenes, and the relative cross-reactivities with roridin A, roridin J, verrucarol, verrucaric acid, satratoxin H, and satratoxin G were 1, 0.41, 0.15, 0.15, and 0.07, respectively. When 16 nonmacrocyclic trichothecenes were tested, only diacetylverrucarol (0.0015) and verrucarol (0.0005) showed minor cross-reactivity. The sensitivity of the enzyme immunoassay for the detection of roridin A was in the range of 5 to 50 ng/ml (0.16 to 1.6 ng per assay).

Among the macrocyclic trichothecenes (36), the roridins appear to be the central members in that further biotransformation leads to most of the other macrocyclic toxins (22). Roridin A (Fig. 1), a cytostatic compound, was isolated from cultures of *Myrothecium verrucaria* and *Myrothecium roridum* (17) and has been produced in fermentation with various *Myrothecium* strains (21). In addition, this toxin could be isolated from the higher plants *Baccharis megapotamica* (23) and *Baccharis coridifolia* (5, 16). This accumulation of macrocyclic trichothecenes was responsible for livestock poisoning in Brazil and Argentina (5, 16). Other veterinary problems have also been attributed to the presence of roridin A in feed (28, 32). In general, the macrocyclic trichothecenes are a serious problem in livestock production (11, 18) and may be a dangerous threat to human health (10). Reliable analytical methods are needed to prevent the economic loss and human health hazards. Unfortunately, gas chromatography and gas chromatography-mass spectrometry (MS), the best techniques to date for determining nonmacrocyclic trichothecenes (34), have serious drawbacks, mainly due to the low volatility of the macrocyclic compounds (2). The known macrocyclic trichothecenes have to be hydrolyzed to their corresponding alcohols and derivatized before analysis (25). Newer techniques such as supercritical fluid chromatography and supercritical fluid chromatography-MS (35) can also be used. These methods are able to detect parts per billion (nanograms per gram) levels of macrocyclic trichothecenes, although they require extensive sample preparation and expensive instrumentation.

The success in developing immunoassays for mycotoxins (for a review, see reference 6) shows an alternate way to screen for these substances. Although much effort has been placed upon the development of immunoassays for nonmacrocyclic trichothecenes, including enzyme immunoassays (EIAs) for T-2 toxin (12, 15, 20, 30, 31), no attempts to produce antibodies against macrocyclic trichothecenes were reported.

In the present report, details are given for the preparation of a roridin A-human serum albumin (HSA) conjugate as an

immunogen, and a roridin A-horseradish peroxidase (HRP) conjugate as an enzyme-linked toxin. Characteristics of antisera obtained after immunization of rabbits and the development of an EIA for the detection of roridin A by using a double-antibody solid phase (1) are described.

MATERIALS AND METHODS

Materials. Roridin A, verrucarol, verrucaric acid, diacetylverrucarol, neosolaniol, T-2, HT-2, diacetoxyscirpenol, scirpentriol, T-2 tetraol, T-2 triol, acetyl-T-2, iso-T-2, T-2 tetraol-tetracetate, 15-acetoxyscirpenol, and deoxynivalenol were purchased from Sigma Chemical Co., Deisenhofen, Federal Republic of Germany. Nivalenol and fusarenon X were obtained from Wako Chemical Co., Neuss, Federal Republic of Germany. Roridin J and satratoxins G and H were provided by the Institute of Biochemistry, University of Mainz, Federal Republic of Germany.

Succinic anhydride, dicyclohexylcarbodiimide (DCCD), *N*-hydroxysuccinimide, *N,N*-dimethylformamide (DMF), HSA, HRP grade VI, fetal calf serum (FCS), 3,3',5,5'-tetramethylbenzidine, and Tween 20 were purchased from Sigma.

Antibody against rabbit immunoglobulin G (IgG) was developed in sheep and purified by affinity chromatography. In brief, the anti-rabbit IgG serum was precipitated with a solution of 70% (vol/vol) ammonium sulfate prepared from a stock solution of saturated ammonium sulfate and dialyzed against phosphate-buffered saline (PBS) (0.01 mol/liter, pH 7.3; phosphate buffer containing 0.1 mol of NaCl per liter). The fractionated antiserum (100 mg) was then added to rabbit IgG coupled to a cyanogen bromide-activated Sepharose 6MB gel (1 g; Sigma) and stirred overnight at 4°C. The gel was recovered by centrifugation and washed with PBS. Specifically bound sheep IgG was eluted with glycine-HCl buffer (0.1 mol/liter, pH 2.5). The pH of the eluted protein was raised immediately to 8.0 with solid Tris, and the eluate was then dialyzed against PBS, quantified by the method of Lowry et al. (26), and stored lyophilized.

All inorganic chemicals and organic solvents were of reagent grade quality or better.

Preparation of roridin A-hemisuccinate. Roridin A (25 mg)

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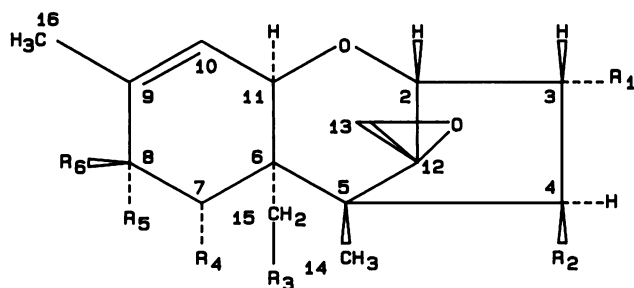


FIG. 2. Structure and numbering system of the trichothecene skeleton. The side chain residues for the nonmacrocytic trichothecenes used in this study are shown in Table 1.

Table 2). The maximum absorbance measured with the preimmune serum as a control was below 0.03 U.

Specificity of competitive EIA. To check the specificity of the roridin A antiserum, 21 different trichothecenes were tested for competition with the roridin A-HRP binding under the conditions of the EIA. All the nonmacrocytic toxins (Fig. 2 and Table 1) were tested in a concentration range of 1 to 60,000 ng/ml, whereas the macrocytic trichothecenes (Fig. 1) were used in a concentration range of 0.1 to 1,000 ng/ml.

RESULTS AND DISCUSSION

To introduce a carboxyl moiety into the molecule, roridin A was reacted with succinic anhydride. After a reaction time of 1 h, more than 90% of the roridin A had been converted to one main derivative and some minor by-products as determined by TLC. The resulting toxin derivative was isolated from the reaction mixture by preparative HPLC. Analysis of the pooled fractions by HPLC with diode array detection showed one substance (retention time, 16.40 min), which was clearly separated from roridin A (retention time, 12.37 min). On the TLC plates only one spot, with an R_f value of 0.80 (R_f for roridin A, 0.72), which gave a positive reaction by the method of Sano et al. (33), could be detected. Since roridin A has two hydroxy groups which can be converted to the corresponding esters by using acetic anhydride (4), the

TABLE 1. Side chain residues (Fig. 2) of the nonmacrocytic trichothecenes tested

Toxin	Side chain ^a					
	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
T-2	OH	Ac	Ac	H	IV	H
Iso-T-2	Ac	OH	Ac	H	IV	H
Acetyl-T-2	Ac	Ac	Ac	H	IV	H
HT-2	OH	OH	Ac	H	IV	H
T-2 triol	OH	OH	OH	H	IV	H
T-2 tetraol	OH	OH	OH	H	OH	H
T-2 tetraol-tetraacetate	Ac	Ac	Ac	H	Ac	H
Diacetoxyscirpenol	OH	Ac	Ac	H	H	H
15-Acetoxyscirpenol	OH	OH	Ac	H	H	H
Scirpentriol	OH	OH	OH	H	H	H
Neosolanol	OH	Ac	Ac	H	OH	H
Verrucarol	H	OH	OH	H	H	H
4,15-Diacetylverrucarol	H	Ac	Ac	H	H	H
Nivalenol	OH	OH	OH	OH	=O	
Deoxynivalenol	OH	H	OH	OH	=O	
Fusarenol X	OH	Ac	OH	OH	=O	

^a Ac, OCOCH₃ (acetate); IV, OCOCH₂CH(CH₃)₂ (isovalerate).

derivative was further characterized by mass spectral and proton nuclear magnetic resonance (¹H nuclear magnetic resonance) analysis. The chemical ionization mass spectrum showed m/z 733 as the protonated molecular ion (the molecular weight of roridin A-dihemisuccinate is 732) and the adduct ions m/z 761 ($M + C_2H_5$)⁺ and m/z 773 ($M + C_3H_5$)⁺, which are typically formed in the methane plasma. The ¹H nuclear magnetic resonance spectra showed significant differences between roridin A and the derivative. The signals corresponding to the protons at C13', C6', and C2' (16) shifted from δ = 3.61, 3.52, and 4.07 ppm (roridin A), respectively, downfield to δ = 4.90, 3.80, and 5.00 ppm (derivative), respectively. In conjunction with the MS data these results confirm that a dihemisuccinate derivative of roridin A was formed.

Toxin-protein conjugates were prepared by coupling the roridin A-hemisuccinate to HSA and HRP by using an activated ester as the reactive intermediate. It has been found that this procedure is adequate for synthesizing both the immunogen and the enzyme-linked toxin (14). No side reactions as described for water-soluble carbodiimides (37) and mixed anhydrides (14) were observed. Based upon UV absorption (Fig. 3), it was calculated that in the synthetic preparation used in this study, approximately 13 and 1 molecules of roridin A-hemisuccinate were bound per molecule of HSA and HRP, respectively.

Antibodies could be detected in the sera of the immunized rabbits as early as 4 weeks after the initial exposure. After one booster injection at week 14, the antibody titer rose until week 20 and then decreased slowly (Table 2). These results are in contrast to the slow response of nonmacrocytic trichothecene immunogens in rabbits, in which moderate levels of specific antibodies could usually be reached only after several booster injections (7, 8). The low immunoge-

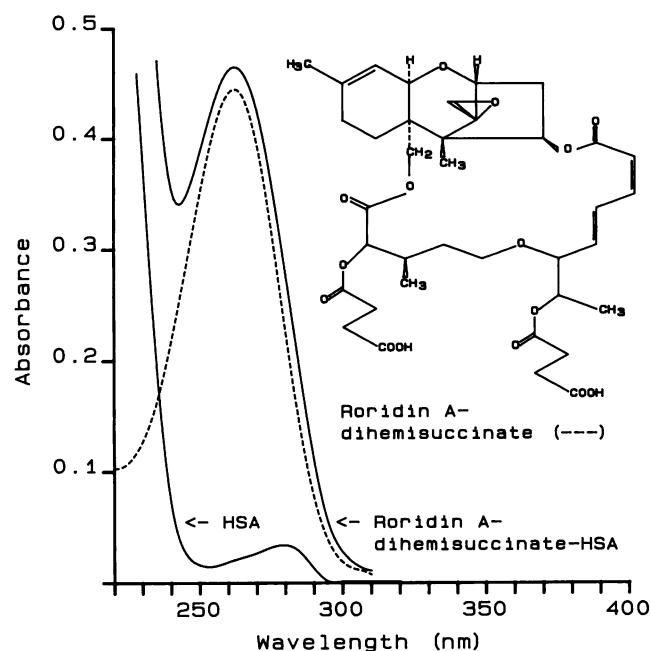


FIG. 3. Comparative absorption scan of HSA (1.27 nmol), roridin A-HSA conjugate (1.27 nmol), and roridin A-dihemisuccinate (16.9 nmol; structure shown). By using the clear difference in A_{260} between the conjugate and an equivalent amount of HSA, it was calculated that approximately 13 molecules of roridin A were bound per molecule of HSA.

TABLE 2. Production of antibody against roridin A

Time (wk) after immunization	Antibody titer (reciprocal of antiserum dilution [10^3]) in rabbit ^a :		
	1	2	3
4	570	100	590
11	680	910	530
14	B	B	B
16	920	620	ND
19	970	810	480
20	1,260	900	490
21	ND	ND	500
22	900	550	ND
24	900	410	ND
28	870	320	ND
30	700	280	ND
33	620	210	ND
36	500	220	ND
37	580	210	ND

^a B, Time of booster injection; ND, not determined.

nicity of the T-2-hemisuccinate-bovine serum albumin conjugate was attributed to the release of the immunosuppressive T-2 toxin from the carrier (19), which might have been due to the relative instability of the T-2-hemisuccinate derivative (38).

By using a double-antibody solid phase (1), a reliable and simple assay with low intraplate variation of color readings could be established. The anti-rabbit IgG serum was purified by affinity chromatography to provide a sufficient IgG-binding capacity (27) and to maximize the signal-to-noise ratio. To minimize intraplate variation of incubation times, the immunological reaction was started by adding the specific antiserum last. Another advantage of the double-antibody solid phase method is that plates coated in this manner could be stored at 4°C for at least 8 weeks without loss of activity. The same precoated plates can be used as the solid phase in every competitive enzyme-labeled-ligand immunoassay if the specific antiserum is developed in rabbits. In

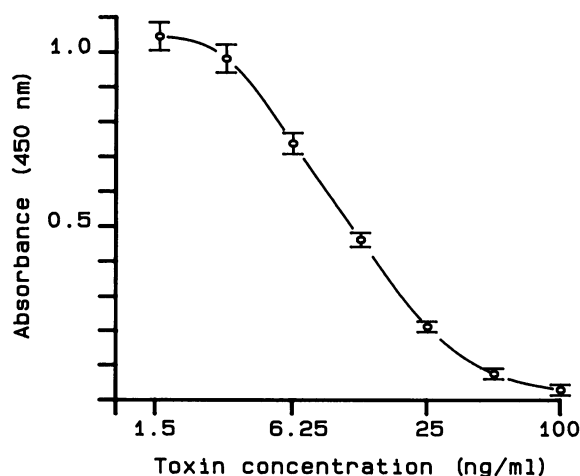


FIG. 4. Standard curve of competitive EIA for detection of roridin A. Microtiter plates (coated with anti-rabbit IgG serum) were incubated with the roridin A-HRP conjugate, increasing concentrations of roridin A, and the specific antiserum. The activity of the bound enzyme was determined as described in the text. The x axis indicates the log of the toxin concentration. Each point represents the mean (\pm standard deviation) of eight replicates.

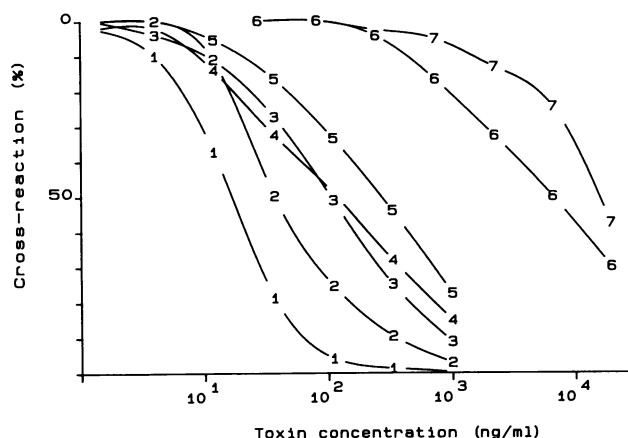


FIG. 5. Effect of roridin A and other trichothecene mycotoxins on binding of roridin A-HRP to antibodies obtained after immunization with roridin A-HSA. Each point represents the mean of four determinations. Symbols: 1, roridin A; 2, roridin J; 3, verrucarol A; 4, satratoxin H; 5, satratoxin G; 6, diacetylverrucarol; 7, verrucarol.

comparison with the direct coating of the specific antiserum, about 10 times less antiserum is needed (27) and the antiserum can be used without any purification step.

Optimal dilutions of antiserum (antiserum from rabbit 1 at week 30 was used throughout this study) and enzyme conjugate for the assay were determined by checkerboard titration and used for the determination of sensitivity and specificity. With Student's *t* test, the detection limit was found at 5 ng/ml (0.16 ng per assay) with a confidence level of 99%. The linear part of the standard curve (Fig. 4) was in the range of 5 to 25 ng/ml, and the intra-assay coefficients of variation ($n = 8$) for standard concentrations (1.5 to 100.0 ng/ml) were usually below 5%.

The specificity of the assay was tested by using different trichothecenes as competitive antigens in the EIA (Fig. 5). The concentrations of different trichothecenes causing 50% inhibition of binding of roridin A-HRP to the antibody are given in Table 3. Diacetylverrucarol and verrucarol were found to be the only nonmacrocytic trichothecenes bound to the antibody. At 60 μ g/ml, the highest concentration tested, none of the other trichothecenes listed in Table 1 showed any significant inhibition. These results indicate that the assay is most specific for the basic trichothecene skeleton combined with the macrocyclic ring system but recognizes to a certain extent the corresponding alcohol (verrucarol). If R1, R4, or R5 (Fig. 2 and Table 1) was substituted, no cross-reaction was observed in this study. These findings were expected because roridin A was coupled to HSA via

TABLE 3. Specificity of roridin A antibody in EIA

Toxin	50% inhibition (μ mol/liter)	% Cross-reactivity relative to roridin A ^a
Roridin A	0.030	100.0
Roridin J	0.074	40.7
Verrucarol A	0.199	15.1
Satratoxin H	0.208	14.8
Satratoxin G	0.459	6.5
Diacetylverrucarol	19.046	0.15
Verrucarol	60.150	0.05

^a (Micromoles of roridin A per liter required for 50% inhibition/micromoles of toxin per liter required for 50% inhibition) \times 100.

the macrocyclic ring, and therefore the basic tetracyclic sesquiterpene structure acted as the immunodominant portion of the toxin-protein conjugate. Structural changes in the vicinity of the coupling site, however, cannot be readily distinguished by the antibody and will show less influence on the immunoreactivity. Nevertheless, the macrocyclic trichothecenes tested in this study showed a certain order of reactivity. The macrocyclic triester verrucarol A was less reactive than roridin J, indicating that the loss of the two-carbon side chain at C6' was recognized by the antibody. Slight changes in structural conformation (roridin J) seem to have a minor influence on immunoreactivity, whereas the six-membered ring of the satratoxins resulted in decreased cross-reactivity, particularly if combined with an additional epoxy group (satratoxin G). Thus, the assay can be considered to be very specific for macrocyclic trichothecenes because none of the most commonly occurring nonmacrocyclic trichothecenes, such as T-2, diacetoxyscirpenol, deoxynivalenol, nivalenol, and fusarenol X (34), was bound by the antibody. It is interesting that in a similar way macrocyclic trichothecenes were not bound by antibodies developed against the nonmacrocyclic toxins T-2 (7) and diacetoxyscirpenol (8). Only antisera against deoxyverrucarol (9) showed weak cross-reactivity with verrucarol A.

The sensitivity and accuracy of the competitive EIA described here seem to be satisfactory for the quantitation of roridin A in feed and may be useful in environmental analytical chemistry. Although it shows substantial cross-reactivity with other macrocyclic trichothecenes, it will differentiate very accurately between macrocyclic and nonmacrocyclic trichothecenes. Since most *Myrothecium* and *Stachybotrys* species can produce different macrocyclic toxins (3, 10, 21), this lack of specificity may be an advantage in monitoring this group of mycotoxins by EIA but will be a disadvantage in analyzing samples for their specific roridin A content.

In light of these observations, confirmation of immunoassay results is still needed for all legal and statutory purposes. Since the principal advantages of immunoassays over conventional methods are the speed and simplicity of the manipulations involved in the procedure, an ideal combination would be the screening of samples by EIA and the confirmation of positive results by chemico-physical methods.

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