Modification of In Vitro Metabolism of T-2 Toxin by Esterase Inhibitors

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Received 8 March 1985/Accepted 16 April 1985

In vitro metabolism of T-2 toxin with S-9 fraction obtained from livers of phenobarbital-treated pigs and rats in the presence of different esterase inhibitors, including NaF, p-hydroxymercuribenzoate, phenylmethylsulfonyl fluoride, eserine sulfate, diisopropylfluorophosphate, and diethyl p-nitrophenyl phosphatase, was studied. The metabolism was completely shifted to the hydroxylation at the C-3' position in the T-2 toxin molecule when esterase inhibitors were present. Diethyl p-nitrophenyl phosphate was found to be the most potent among six esterase inhibitors tested. In the presence of $10^{-4}$ M diethyl p-nitrophenyl phosphate, 3'-hydroxy-T-2 toxin was the only metabolite detected. Similar results were obtained when other T-2-related metabolites were tested. The yield of conversion of T-2 toxin, acetyl T-2 toxin, HT-2 toxin and T-2 triol to their respective 3'-hydroxyl derivatives were 82, 73, 72, and 75%, respectively.

MATERIALS AND METHODS

Chemicals. T-2 toxin was prepared as described previously (14). HT-2, T-2 triol, and T-2 tetraol were prepared by mild alkaline hydrolysis of T-2 toxin (14). Tritium-labeled T-2 toxin at a specific activity of 19 Ci/mmol was prepared by the procedure of Wallace et al. (13) as modified by Chu et al. (4). Ac-T-2 was synthesized from T-2 toxin by acetylation in acetic anhydride-pyridine (1). Glucose 6-phosphate, NADP+, NaF, p-hydroxymercuribenzoate, phenylmethylsulfonyl fluoride, eserine sulfate, diisopropylfluorophosphate, and diethyl p-nitrophenyl phosphate (DENP) were purchased from Sigma Chemical Co., St. Louis, Mo. Amberlite XAD-2, (20-25 mesh) was obtained from Eastman Kodak Co., Rochester, N.Y. The resin was washed by swirling and decanting three times with 3 volumes of acetone followed by methanol and distilled water and stored in water at 4°C. Florisil (60-100 mesh) was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. All other chemicals or solvents were either chemically pure or reagent grade.

Preparation of liver homogenate S-9 fraction. Male Sprague-Dawley rats (Sprague-Dawley, Madison, Wis.) weighing about 200 g and male pigs (University of Wisconsin Farm, Madison, Wis.) weighing about 100 kg were used for the preparation of liver S-9 fractions. Rats were given 0.1% phenobarbital sodium in drinking water for 5 days before sacrifice. Pigs received 4 g of phenobarbital sodium, dissolved in 10 ml of 0.8% NaCl, by intramuscular injection for 4 consecutive days. All the animals were sacrificed after the enzyme induction treatments. Liver from a pig without previous treatment was used for comparison. Livers were rinsed with cold 0.25 M sodium phosphate buffer (pH 7.4) and homogenized in 4 volumes of the same buffer. A Waring blender and a glass-Teflon homogenizer were used for pig and rat livers, respectively. The homogenate was centrifuged at 9,000 x g for 10 min at 4°C. The supernatant solution was filtered through glass wool and is referred to as S-9. The protein concentrations of these preparations were determined by the procedure of Lowry et al. (7).

Metabolic studies. To a 25-ml Erlenmeyer flask the following reactants were added: enzyme inhibitor, if any; 160 μmol...
of MgCl₂ and 660 μmol of KCl in 200 μl of sodium phosphate buffer; 5 ml of S-9; 100 μmol of glucose 6-phosphate in 50 μl of sodium phosphate buffer; 10 μmol of NADP⁺ in 50 μl of sodium phosphate buffer; and 6.4 μmol of T-2 toxin with a trace amount of ³H-labeled T-2 toxin (2.88 × 10⁴ dpm) in 200 μl of ethanol. The reaction mixture was incubated with shaking (105 strokes per min) and aeration at 37°C for 1 h, and then the reaction was terminated by dilution with distilled water to a total volume of 10 ml, of which a 20-μl sample was pipetted and used for liquid scintillation counting. The rest of the mixture was introduced into an Amberlite XAD-2 column (1.0 by 10 cm) at a flow rate of about 1 drop per s by regulating the opening of the Teflon stopcock. The column was then washed with 25 ml of distilled water, and the toxin as well as the metabolites were eluted from the column with 20 ml of acetone. In the initial experiments, all fractions were saved, and the radioactivity of each portion was measured. Since the radioactivity was only found in the acetone fraction, only the acetone eluate was saved for further studies. Generally, the acetone eluate was evaporated under a stream of nitrogen gas to approximately 2 to 6 ml, from which 5-μl samples were taken for radioactivity measurement and thin-layer chromatography (TLC) analysis.

TLC analysis. TLC was carried out on plastic sheets precoated with silica gel G 60 (thickness, 0.2 mm; E. Merck, Darmstadt, Federal Republic of Germany). Standards of T-2, HT-2, T-2 triol, and T-2 tetraol as well as the samples were spotted side by side. The sheet was developed with chloroform-acetone (3:2 [vol/vol]). Some of the TLC sheets were charred with 10% H₂SO₄ in ethanol and heated to 110°C for localizing the spots. A corresponding area of the spot from an unspayed sheet was cut and put into a vial for liquid scintillation counting.

Production of 3'-OH-T-2 toxin. For the production of microgram quantities of 3'-OH-T-2 toxin, 9.7 μmol of DENP in 420 μl of ethanol was placed in a 500-ml Erlenmeyer flask, followed by addition of 3.2 mmol of MgCl₂, 13.2 mmol of KCl, 100 ml of pig liver S-9 (from a phenobarbital sodium-treated pig; 27 mg of protein per ml), 2 mmol of glucose 6-phosphate, 0.2 mmol of NADP⁺, and 60 mg (129 μmol) of T-2 toxin in 0.1 ml of dimethylsulfoxide and 2 ml of ethanol. The reaction was carried out at 37°C for 1 h by shaking and aeration and terminated by directly applying it to an Amberlite XAD-2 column (1.9 by 19 cm). After washing with 150 ml of distilled water, unreacted T-2 toxin and metabolites were eluted from the column with 150 ml of acetone. The acetone eluate was rotary evaporated to a few milliliters. Distilled water (10 ml) was added to the residue, which was then extracted with two 30-ml portions of CH₂Cl₂. The combined CH₂Cl₂ extract was concentrated in a rotary evaporator to an oil product, which was subsequently redisolved in a small amount of CHCl₃ and chromatographed on a 5-g Florisil column (1 by 10 cm). Stepwise elution with solvents containing CHCl₃ and different concentrations of methanol (1 to 10%) was performed by the method of Yoshizawa et al. (17). The eluate with 2% methanol in CHCl₃ gave a single TLC spot at Rf 0.45 (chloroform-acetone, 3:2 [vol/vol]) compared with T-2 toxin at Rf 0.60 and HT-2 toxin at Rf 0.25. This fraction weighed 51 mg after it was evaporated to dryness. The structure of 3'-OH-T-2 toxin was confirmed by nuclear magnetic resonance (NMR).

Production of other 3'-hydroxy metabolites of T-2 toxin. Conditions for the production of 3'-OH-Ac-T-2, 3'-OH-HT-2, and 3'-OH-T-2 triol were the same as those for the preparation of 3'-OH-T-2 except that Ac-T-2, HT-2 toxin, and T-2 triol were used as the substrates. Separation of different metabolites was achieved by a Florisil (5-g) column with stepwise elution in which 3'-OH-Ac-T-2, 3'-OH-HT-2, and 3'-OH-T-2 triol were eluted from the column with 1, 5, and 7% methanol in CHCl₃, respectively.

Determination of radioactivity. A sample, generally less than 1 ml, or a strip cut from the TLC sheet was mixed with 8 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and counted on a Beckman LS 5800 liquid scintillation system.

Spectroscopy. Mass spectra of the purified compounds were determined in a Finnigan 4500 GC-MS spectrometer. Proton NMR spectra were measured in a Bruker 270 NMR spectrometer.

RESULTS AND DISCUSSION

In the initial experiments, several well-known esterase inhibitors were selected to test their inhibitory effect on the deacetylation of T-2 toxin by pig liver S-9. At concentrations of 6 × 10⁻³ and 1 × 10⁻⁴ M respectively, NaF and p-chloromercuribenzoate showed no effect as compared with the controls. Formation of HT-2 toxin, however, was markedly inhibited by several other inhibitors. At concentrations of 2.5 × 10⁻², 1 × 10⁻³, 1 × 10⁻⁴, and 1 × 10⁻⁴ M, phenylmethylsulfonyl fluoride, eserine sulfate, diisopropylfluorophosphate, and DENP showed 55, 69, 87, and 99.5% inhibition, respectively, of the deacetylation reaction.

Because DENP was found to be the most effective inhibitor, subsequent studies were concentrated on this inhibitor.
Representative results for the effect of DENP on T-2 metabolism as measured by TLC and radioactivity counting of the metabolites are shown in Fig. 2. The \( R_f \) values for T-2, 3'-OH-T-2, HT-2, T-2 triol, and T-2 tetrool in the solvent system of chloroform-acetone (3:2 [vol/vol]) were found to be 0.6, 0.45, 0.25, 0.13, and 0.05, respectively. These results are consistent with those reported by Yoshizawa et al. (17). Incubation of T-2 toxin with pig liver S-9 and other cofactors at 37°C for 1 h resulted in the total disappearance of this toxin. Essentially all the toxin hydrolyzed to HT-2 toxin (67%; Fig. 2, lane b). This observation agrees well with the results obtained by other investigators (5, 9). When DENP (10^{-4} M) was present in the reaction mixture, hydrolysis of T-2 toxin was almost completely blocked. Such modification promoted the hydroxylation reaction and thus led to the production of more 3'-OH-T-2 (31%; Fig. 2, lane c). When the toxin was incubated with DENP and S-9 fraction prepared from the liver of a pig that had been treated with phenobarbital, the hydroxylation reaction became a predominant pathway; as much as 81% of the T-2 toxin was converted to 3'-OH-T-2 (Fig. 2, lane e). Similar results were obtained when S-9 fraction obtained from livers of phenobarbital-treated rats was used as the enzyme source (Fig. 2, lane f). In a typical large-scale experiment, 51 mg of 3'-OH-T-2 was obtained from 60 mg of T-2 toxin. This yield (82%) is considerably higher than that recently reported by Yoshizawa et al. (24.9%) (17). Because of such a high yield of conversion and the lack of interference metabolites, purification of this compound became much easier. Mass spectral and NMR data of the 3'-OH-T-2 were identical to those reported previously (17).

By the same approach, 3'-OH-Ac-T-2, 3'-OH-HT-2, and 3'-OH-T-2 triol were prepared after incubation of Ac-T-2, HT-2, and T-2 triol, respectively, with pig liver S-9 (from a phenobarbital-treated pig) and the NADP⁺ regeneration system in the presence of DENP.

The product isolated after incubation with Ac-T-2 was identified as 3'-OH-Ac-T-2 and had the same \( R_f \) values as T-2 toxin in TLC in several solvent systems: chloroform-acetone (3:2 [vol/vol]) at 0.59, toluene-ethyl acetate (1:3 [vol/vol]) at 0.4, and ethyl acetate-acetone-methanol (50:50:1 [vol/vol/vol]) at 0.72. The yield (73%) for the pure compound was 44 mg from 60 mg of starting Ac-T-2. Methane chemical ionization mass spectrum showed a strong M + 1 peak at 525. The \(^1\)H NMR spectrum of this compound is shown in Fig. 3A. A typical diagnostic resonance due to gem-dimethyl proton of the C-8 substituent was observed at 1.298 as a singlet rather than a doublet resonance at 0.96 in T-2 toxin. Three strong singlets were observed at 2.169, 2.111, and 2.087, which correspond to three acetate ester methyl groups. Although 3'-OH-Ac-T-2 has been chemically synthesized from 3-acetyl-neosolaniol (16), the present in vitro method offers an alternate approach for the production of this compound with a good yield.

In the HT-2 toxin experiment, 18 mg of 3'-OH-HT-2 was obtained from 25 mg of HT-2 toxin. This yield of conversion (72%) was considerably higher than that obtained in a previous investigation (17) in which only 3.2% of the HT-2 was converted to 3'-OH-HT-2 when mouse hepatic S-9 fraction was used. Methane chemical ionization mass spectrum showed an M + 1 peak at 441. The \(^1\)H NMR spectrum of this compound (Fig. 3B) is identical to that obtained by Yoshizawa et al. (18).

A high yield of 3'-OH-T-2 triol (75%) was obtained also after incubation of T-2 triol with S-9 fraction in the presence of DENP. In a typical experiment, 24 mg of pure 3'-OH-T-2 triol was obtained from 32 mg of T-2 triol. Methane chemical ionization mass spectrum of its acetylated product showed...
In the present study, we found that the in vitro metabolism of T-2 toxin could be modified by several esterase inhibitors. Inhibition of deacetylation of T-2 toxin by eserine and diisopropylfluorophosphate was also observed by Ohta et al. (9). Since we and also Ohta et al. (9) observed that the organophosphate types of inhibitors are more potent, deacetylation of T-2 toxin is more likely caused by acetylcholine-type enzymes rather than by the general nonspecific esterases (3). Ohta et al. also observed that the acetyl group at the C-4 position of trichothecenes is more easily subjected to deacetylation by the enzymes than are acetyl groups at other positions. Although from a practical point of view, the results obtained from the present study offer a very efficient method for the production of large quantities of these hydroxylated metabolites for other studies, our results also imply that the toxicity of T-2 toxin might be altered when such inhibitors are present in foods and feed. In more complex environments, such as fields in which organophosphates have been used, such inhibitors may also affect the biotransformation of T-2 toxin and thus alter the ratio of contamination of T-2 toxin and HT-2 toxin in agricultural commodities.

FIG. 3. The 270-MHz 1H NMR spectra of 3'-OH-Ac-T-2 (A), 3'-OH-HT-2 (B), and 3'-OH-T-2 triol (C) in CDCl3, with tetramethylsilane as the internal standard.

ACKNOWLEDGMENTS

This work was supported by grant NC-129 from the College of Agricultural and Life Sciences, the University of Wisconsin at Madison; Public Health Service grant CA-15064 from the National Cancer Institute; and contract DAMD-82-C-2021 from the U.S. Army Medical Research and Development Command of the Department of Defense.

We thank Guangshi Zhang for his assistance in the NMR and mass spectrum analyses and Susan Schubring for her help in the preparation of the manuscript.

LITERATURE CITED


