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METABOLISM OF T-2 TOXIN BY RAT LIVER CARBOXYLESTERASE

HELGE JOHNSEN, ERLING ODDEN, ØYSTEIN LIE,* BJØRN A. JOHNSEN and FRODE FONNUM
Norwegian Defence Research Establishment, Division for Environmental Toxicology, N-2007 Kjeller, Norway

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Abstract—The trichothecene T-2 toxin was rapidly hydrolyzed by rat liver microsomal fraction into HT-2 toxin which was the main metabolite. The metabolism was completely blocked by paraoxon, a serine esterase inhibitor, but not affected by EDTA or 4-hydroxy mercury benzoate, inhibitors of arylesterase and esterases containing SH-group in active site, respectively. Among the serine esterases carboxylesterase (EC 3.1.1.1), but not cholinesterase (EC 3.1.1.8) hydrolysed T-2 toxin to HT-2 toxin. Carboxylesterase activity from liver microsomes was separated into at least five different isoenzymes by isoelectric focusing, and only the isoenzyme of pI 5.4 was able to hydrolyse T-2 toxin to HT-2 toxin.

The toxicity of T-2 toxin in mice was enhanced by pre-treatment with tri-o-cresyl phosphate (TOCP), a specific carboxylesterase inhibitor. This confirms the importance of carboxylesterase in detoxification of trichothecenes.

T-2 toxin and related trichothecenes are potent cytotoxic and immunosuppressive mycotoxins produced by various species of Fusarium. The toxins may cause dermal necrosis, hemorrhaging, leukopenia and inhibition of protein synthesis in various animals and biological systems [1–3]. T-2 toxin (Fig. 1) is one of the most important trichothecene mycotoxins occurring naturally in various agricultural products [4, 5], and appears to be an important human health problem.

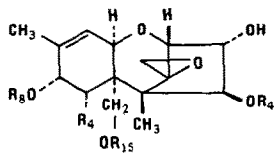
It has been shown that T-2 toxin administered orally to rodents, chickens and a lactating cow, was rapidly transformed into various metabolites and eliminated without accumulation in any organ [6–8].

In mouse and rat liver homogenates, T-2 toxin was rapidly hydrolyzed to HT-2 toxin, which was converted further into T-2 tetraol via 4-deacetyl neosolaniol [10, 11]. Other authors, however, have reported HT-2 toxin as the only metabolite of T-2 toxin produced in rat liver microsomal fraction [12, 13]. *In vitro* formation of 3′-hydroxy T-2 and 3′-hydroxy HT-2 toxins from T-2 toxin by liver homogenates from mice pretreated with phenobarbital and from monkeys by adding NADPH have been reported, indicating that a cytochrome P-450 is catalyzing the hydroxylation at C-3′ position of T-2 and HT-2 toxin [11]. Two of the metabolites found in the lactating cow were also identified as 3′-hydroxy T-2 and 3′-hydroxy HT-2 [9].

Ohta *et al.* [13] found that hydrolysis of T-2 toxin was inhibited by eserine and DFP. They also observed a poor hydrolytic activity in intestinal mucous and serum. On the basis of these findings they suggested that nonspecific carboxylesterase most likely catalyzed the conversion of T-2 toxin to HT-2 toxin. By structure-activity studies they suggested that the esterase selectively hydrolyzed the C-4 acetyl residue of trichothecenes, and that substituents at C-3 and C-8 were essential for the enzymatic hydrolysis of the C-4 acetyl residue [12]. In the present paper the hydrolysis of T-2 toxin by rat liver microsome preparation has been studied in detail. By use of several specific esterase inhibitors, the properties of the enzyme responsible for the hydrolytic activity have been characterized. Such studies strongly indicate that the activity can be accounted for by a carboxylesterase. Isoelectric focusing separated the carboxylesterase activity into five different isoenzymes, and their activity to T-2 toxin was studied.

MATERIALS AND METHODS

Chemicals. 3′-Hydroxy T-2 and 3′-hydroxy HT-2 toxins were generous gifts from C. J. Mirocha, MA, U.S.A. T-2 toxin, acetyl T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol (Fig. 1) and 4-nitrophenyl acetate, 4-nitrophenyl butyrate, bis-4-nitrophenyl phosphate and carboxylesterase (EC



TRICHOTHECENE	R_4	R_8	R_{15}
T-2	CH_3CO	$(CH_3)_2CH\ CH_2CO$	CH_3CO
HT-2	H	$(CH_3)_2CH\ CH_2CO$	CH_3CO
4-DEACETYL-NEOSOLANIOL	H	H	CH_3CO
T-2 TETRAOL	H	H	H

Fig. 1. Chemical structure of trichothecene mycotoxins.

* Present address: National Veterinary Institute, DEP., 0033 Oslo 1, Norway.

3.1.1.1) from porcine liver were all purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Horse serum cholinesterase (EC 3.1.1.8) and paraoxon were from Koch-Light Laboratories, U.K., saponin from Merck, Darmstadt, F.R.G. and tri-*o*-cresyl phosphate (TOCP) from K and K, JCN Pharmaceuticals Inc., U.S.A. Sylon BTZ from Supelco Inc., PA, U.S.A. was used as trimethylsilyl derivatizing reagent for gas chromatography of trichothecenes.

Ultrogel AcA34, bead size (swollen) 60–140 μm for gelfiltration was purchased from LKB, France, while polyacrylamide electrofocusing gel (Bio-Lyte) and ampholytes (Bio-Lyte) were from Bio-Rad Laboratories, CA, U.S.A. Soman was synthesized in our laboratory, and methyl-[1-^{14}C]butyrate synthesized as described [14].

Preparation of liver fractions. Livers 10–12 g, from male Wistar rats (250–300 g) were rinsed with ice-cold 50 mM Tris pH 7.5, containing in 0.1 M NaCl and homogenized in 4 vol. of the same buffer with glass–Teflon homogenizer. The homogenate was filtered through glass wool to remove fat and centrifuged at 9000 *g* for 20 min at 4°. The supernatant, S9 fraction, was further centrifuged at 100,000 *g* for 60 min at 4°. The high speed pellet was resuspended in an equal volume of 50 mM Tris pH 7.5 and constituted the microsomal fraction. The high speed supernatant was fractionated by ammonium sulfate precipitation. The hydrolytic activities were mainly precipitated in the fraction from 35–70% ammonium sulfate saturation. This precipitate was resuspended in a small volume of 50 mM Tris pH 7.5, and referred to as the cytosol fraction.

For gelfiltration and isoelectric focusing the microsomal fractions were solubilized by mixing with 1% saponin for 1 hr at 4° and centrifuged at 100,000 *g* for 60 min. The 35–70% ammonium sulfate fraction of the high speed supernatant was resuspended in a small volume of 50 mM Tris pH 8.0 for gelfiltration and 10 mM Tris pH 8.0 for isoelectric focusing. This is called microsomal enzyme fraction.

Enzyme assay. Carboxylesterase assays were performed at pH 7.8 and 30° with the substrates methyl [1-^{14}C]butyrate (1.76 mM), 4-nitrophenyl butyrate (2 mM) and 4-nitrophenyl acetate (0.25 mM) as previously described [14]. Liberated [1-^{14}C]butyrate was measured by scintillation counting and 4-nitrophenol was determined spectrophotometrically at 400 nm [14]. In some experiments we have also used acetanilide as substrate [15].

Gel filtration. The microsomal enzyme fraction containing 100–150 mg protein was applied to a gel filtration column (Ultrogel AcA34, 35 × 25 cm) equilibrated with 50 mM Tris pH 8.0. The sample was eluted at a rate of 0.3 ml/min and fractions collected every 5 min. The fractions were then analyzed for carboxylesterase activity, and peak activities were analyzed for T-2 hydrolysis.

Isoelectric focusing. The microsomal enzyme fraction was desalted by eluting through Sephadex G-25M (Pharmacia PD-10 column, 5 × 1.5 cm) equilibrated with distilled water. Between 5 and 8 ml of the eluate corresponding to 100–150 mg protein was mixed with 90 ml of Bio-Lyte electrofocusing gel slurry containing 3% of Bio-Lyte ampholytes pH 3/10:5/7 (2:3). The slurry was then applied onto a flat bed tray (30 × 12.5 cm) and dried by airstream. The electrolytes were 0.5 M H_3PO_4 for the anode and 0.5 M NaOH for the cathode. A constant power of 25 W was applied for 16 hr at 5°. Harvesting was performed by a grid with 0.7 cm sections, proteins eluted from the gel by mixing with 1 ml distilled water and centrifugation. The fractions were analyzed for carboxylesterase activity and T-2 hydrolyzing activity.

Incubation of T-2 toxin. Aliquots of 1 ml of 10% microsome preparation and 1 ml of cytosol preparation were preincubated with enzyme inhibitors for 30 min at 37° and pH 7.5. To this incubation mixture was added 0.5 μmole T-2, and the incubation continued for 10 and 60 min, respectively. Samples of 1 ml from gel filtration and 0.5 ml from isoelectric focusing mixed with 0.5 ml 0.1 M Tris pH 7.5 were incubated with 0.5 μmole T-2 toxin for 2 hr at 37° and pH 7.5.

The reaction was stopped by adding 2 ml ice-cold 0.6 M perchloric acid and 125 μg diacetoxyscirpenol as internal standard. The sample was centrifuged at 15,000 *g* for 10 min, and the supernatant was treated with 180 μl 7.5 M potassium acetate, recentrifuged and applied onto Amberlite XAD-2 resin column (1 g, 1 × 20 cm). The column was washed with 20 ml of water and eluted by 20 ml of methanol:water (9:1 vol/vol). The methanol eluate was evaporated to dryness and dissolved in 0.5 ml acetone. Aliquots of 50 μl of the acetone extract was mixed with 25 μl Sylon BTZ for trimethylsilyl derivatizing for gas chromatography. Recovery for T-2 toxin and HT-2 toxin by the whole method was about 90% and 30–40% for T-2 tetraol.

In cases where only the metabolism of T-2 and HT-2 was studied, a faster isolation procedure was used by means of Clin-Elute diatomaceous earth column (1 g, 2.5 × 1.2 cm). Recovery of T-2 and HT-2 by this procedure was 70–80%. One millilitre of the sample was applied to the column and it was eluted with 5 ml chloroform. The chloroform extract was evaporated to dryness and dissolved in 500 μl acetone and aliquots of 50 μl were mixed with 25 μl Sylon BTZ for derivatizing.

Gas chromatographic analysis of trichothecenes. Gas chromatography was performed by a Packard Becker 438 equipped with a mass spectrometer (LKB 2091) for identification, or flame ionization detector for quantification of trichothecene metabolites. A glass column (1.2 m × 2 mm I.D.) packed with 3% OV-17 on Supelcoport 80–100 mesh was used under the following conditions: oven temperature programmed from 200° to 275° at 15°/min, injector and detector temperature 300°, flow rate of helium, hydrogen and air; 30, 25 and 250 ml/min respectively. The operating conditions for mass spectrometry were: ionization energy 70 eV or 12 eV, accelerating voltage 3.5 kV. Quantification of metabolites was based on standard curves and diacetoxyscirpenol as internal standard.

RESULTS

T-2 toxin incubated with rat liver microsomal fraction was completely metabolized to HT-2 toxin after

Table 1. Effect of esterase inhibitors on rat liver metabolism of T-2 toxin

Fraction inhibitor	Carboxylesterase activity (μmole 4-NPB*) mg prot × hr		T-2 metabolism (% HT-2 PRODUCED) mg prot × 10 min	
Microsomes (P2)	400 ± 62	(100%)	44 ± 4	(100%)
Microsomes TOCP† (200 mg/kg)	89 ± 18	(22%)	27 ± 1	(60%)
Microsomes BPNP‡ (10^{-4} M)	51 ± 5	(13%)	19 ± 3	(44%)
Microsomes SOMAN (10^{-5} M)	11 ± 2	(3%)	18 ± 5	(40%)
Microsomes SOMAN (10^{-4} M)	9 ± 0	(2%)	2 ± 0	(4%)
Microsomes PARAOXON (10^{-4} M)	0	(0%)	0	(0%)
Microsomes EDTA (10^{-3} M)	409	(100%)	43 ± 1	(100%)
Microsomes MERCURY BENZOATE (10^{-3} M)	338	(84%)	44 ± 1	(100%)
Cytosol (S2)	9 ± 3	(100%)	0.19 ± 0.03	(100%)
Cytosol TOCP (200 mg/kg)	3 ± 0.3	(33%)	0.13 ± 0.02	(70%)
Plasma	3.8 ± 0.3	(100%)	0	(0%)
Plasma TOPC (200 mg/kg)	0.5 ± 0.2	(13%)	0	(0%)

The numbers are mean values ± S.D. of 4–8 rats.
Inhibitors were preincubated at 37° for 30 min before incubation with 0.5 mM and T-2 toxin.
* 4-nitrophenylbutyrate.
† Tri-*o*-cresyl phosphate.
‡ Bis-4-nitrophenylphosphate.

60 min, and no other metabolites were found by GC-MS under the experimental conditions used. In rat liver cytosol fraction the T-2 to HT-2 metabolic activity was much lower, and in plasma no metabolism was found (Table 1).

The metabolic activity was completely blocked by paraoxon (10^{-4} M), which is known to inhibit serine esterases. No effect, however, was seen by EDTA (10^{-3} M), an aryl esterase inhibitor [16, 17], or 4-hydroxy mercury benzoate (10^{-3} M), which inhibit esterases with SH-group in the active site, such as in phosphoryl phosphatases [18]. It is apparent that the metabolism of T-2 toxin is less sensitive than the 4-nitrophenyl butyrate hydrolysis towards organophosphorus inhibitors such as soman (10^{-5} M) and bis-4-nitrophenyl phosphate (10^{-4} M). At higher concentration of soman (10^{-4} M) the metabolic activity was completely inhibited. We therefore concluded that the metabolism of T-2 toxin corresponded to a group of serine-esterases.

Of the commercially available preparations of serine esterases shown in Table 2, carboxylesterase isolated from porcine liver, but not cholinesterase isolated from horse serum, hydrolized T-2 toxin.

Because of the high cost of trichothecenes, the effect of carboxylesterase inhibition on toxicity of T-2 toxin was studied in mice. As shown in Table 3, the toxicity of T-2 toxin is increased by selective inhibition of carboxylesterase *in vivo* by tri-*o*-cresyl phosphate (TOCP) [19], indicating the importance of carboxylesterase for detoxification of T-2 toxin. TOCP is not toxic to mice at this dosage. The carboxylesterase activity in liver homogenates of TOCP-treated animals, measured by the 3 substrates (4-nitrophenyl butyrate, 4-nitrophenyl acetate and methyl [1-^{14}C]butyrate), was only 10–30% of normal activity.

Carboxylesterase is a heterogenous group of enzymes consisting of several isoenzymes [20]. From Table 1, it is apparent that the isoenzyme mainly

Table 2. Hydrolytic activity of commercial serine esterases

Serine esterase	Incubation time (min)	% HT-2 produced of added T-2 toxin
Cholinesterase*−5 mg/ml	60	0
Carboxylesterase†		
−1 mg/ml	10	8
−1 mg/ml	30	26
−2.5 mg/ml	10	16
−2.5 mg/ml	30	60

Enzymes were dissolved in 50 mM Tris pH 7.5 and incubated with 0.5 mM T-2 toxin at 37°

* Cholinesterase (horse serum): $\frac{20\ \mu\text{mole butyryl thiocholine}}{\text{mg material} \cdot \text{min}}$.

† Carboxylesterase (porcine liver): $\frac{160\ \mu\text{mole ethyl butyrate}}{\text{mg protein} \cdot \text{min}}$.

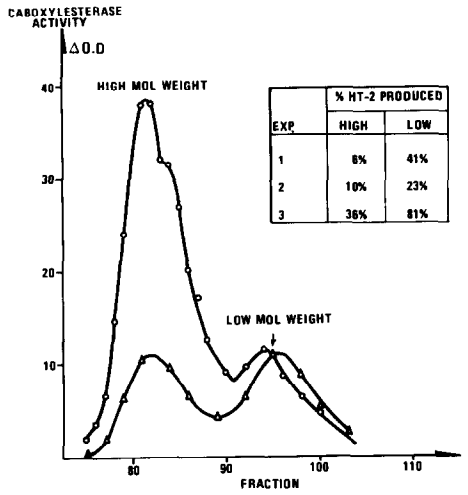


Fig. 2. Gel filtration of rat liver carboxylesterase on Ultrogel AcA34. The column was eluted with 50 mM Tris pH 8.0 at 0.3 ml/min and fractions collected every 5 min. Fractions were analyzed for 4-nitrophenyl butyrate (○——○) and 4-nitrophenylacetate (△——△) activity and peak activities were analyzed for T-2 hydrolyzing activity.

responsible for T-2 to HT-2 metabolism is characterized by being less sensitive both to low concentration of soman (10^{-5} M) and bis-4-nitrophenyl phosphate (10^{-5} M). Separation of carboxylesterase isoenzymes into one high and one low molecular weight fraction identified by the substrates 4-nitrophenyl butyrate and 4-nitrophenyl acetate is shown in Fig. 2. The main hydrolytic activity for T-2 toxin was found in the low molecular weight fraction. The remaining activity in the high molecular weight fraction may be contamination due to incomplete separation of the two peaks.

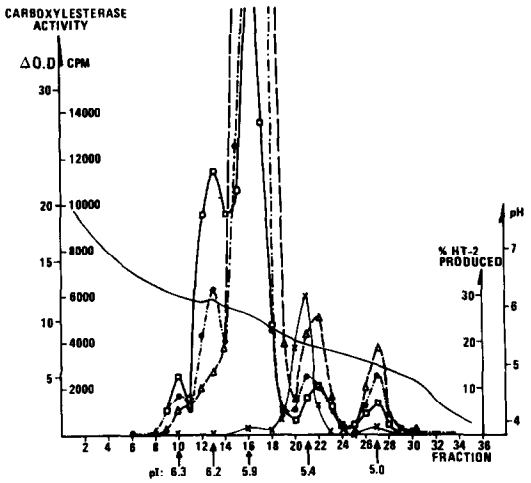


Fig. 3. Preparative flat bed isoelectric focusing of rat liver carboxylesterase on polyacrylamide gel. The fractions were analyzed for carboxylesterase activity by the substrates; 4-nitrophenyl butyrate (●—·—·—●), 4-nitrophenyl acetate (□——□) and methyl[1-^{14}C]butyrate (△——△). The five different carboxylesterase isoenzyme were analyzed for T-2 hydrolyzing activity (×——×).

Separation of the isoenzymes by isoelectric focusing is shown in Fig. 3. Carboxylesterase was separated into 5 different isoenzymes according to their isoelectric point (pI), and identified by the 3 different substrates. The isoenzyme pI 5.9 is by far the most active isoenzyme with respect to hydrolysis of all 3 different substrates, but no T-2 hydrolyzing activity was found. It is known that this isoenzyme constitutes the high molecular weight fraction (180,000 MW), whereas the 4 other constitute the low molecular weight fraction (60,000 MW) [21]. It is apparent from Fig. 3 that T-2 to HT-2 metabolism in rat liver is performed by the isoenzyme pI 5.4 only. Isoenzyme pI 5.4 was the only isoenzyme which displayed high activity towards the substrate acetanilide (data not shown). However, as can be seen from the figure, there is a slight difference between the peak activities of 4-nitrophenyl acetate, methyl-[1-^{14}C]butyrate and the peak activity to T-2 hydrolysis, indicating a possible microheterogeneity of isoenzyme pI 5.4. We attempted to resolve this peak by refocusing in a pH gradient 4.5–6, but the peaks achieved were too close to allow any separation by flat bed isoelectric focusing. Separate experiments showed that the enzyme activity of isoenzyme pI 5.4 towards 4-nitrophenyl butyrate, 4-nitrophenyl acetate, methyl[1-^{14}C]butyrate and T-2 toxin was inhibited to the same extent by soman ($5 \cdot 10^{-6}$ M).

DISCUSSION

We have found that in rat liver microsomal fraction, T-2 toxin is metabolized to HT-2 toxin and that the metabolism is performed mainly by a carboxylesterase at pI 5.4. We did not, even after extended incubation, find any other metabolites under our incubation conditions.

We conclude that the metabolism of T-2 to HT-2 is performed by a serine esterase, since it is completely blocked by paraoxon. We could exclude an aryl esterase and phosphoryl phosphatase, since it was not inhibited by EDTA or 4-hydroxy mercury benzoate. Among the serine esterases shown in Table 2, carboxylesterase but not cholinesterase hydrolyses T-2 to HT-2, and carboxylesterase therefore seems to be the most likely candidate for the metabolism of T-2 to HT-2. The results of Ohta *et al.* [13] by use of eserine for inhibition is not conclusive in differentiation between cholinesterase and other esterases.

Table 3. Effect of a carboxylesterase inhibitor on the toxicity of T-2 toxin in mice

T-2 TOXIN (mg/kg I.V.)	% Survival	
	Control	TOCP (100 mg/kg)
0.5	100	100
1.0	100	100
2.0	100	0
3.0	70	0
4.0	10	0

Each group consisted of 10 animals

Of particular interest was the fact that much lower activity than expected from carboxylesterase activity was derived from the cytosol fraction and that no metabolic activity was found with rat plasma even after incubation for 60 min. These facts strongly indicate that the metabolic activity could not be accounted for by the whole carboxylesterase group. Thus our conclusion is an extension of the findings by Ohta *et al.* [13], who reached their conclusions only on the basis of results obtained with DFP and eserine. Carboxylesterase is a heterogeneous group of enzymes consisting of several isoenzymes [20, 21]. Carboxylesterase in liver microsomes were separated into 5 different isoenzymes according to their isoelectric point. The isoenzymes revealed different substrate specificity, and the isoenzyme of pH 5.4 was the only isoenzyme able to hydrolyse T-2 toxin to HT-2 toxin. This isoenzyme was found by us to exhibit activity towards acetanilide in agreement with the findings by Mentlein and Heymann [15].

Under our experimental conditions we did not find any 4-deacetyl neosolaniol or T-2 tetraol, the further deacetylated products of HT-2. This is in contrast to the findings of Yoshizawa *et al.* [10] who detected both 4-deacetyl neosolaniol and T-2 tetraol in S9 fractions from rat liver. In their study they stopped the reaction by putting the test tube into a waterbath at 85°. This gradual heating up to 85° will, however, not immediately denaturate the proteins. Since carboxylesterases are heat stable at 50–60° the heating could possibly accelerate the hydrolysis. The heating could also provide sufficient energy to start a further hydrolysis of HT-2. In agreement with our findings Ohta *et al.* [13] and Ellison *et al.* [22] did not find any other metabolites than T-2 and HT-2 although their clean-up procedure could be criticized for being insensitive to the polar metabolites of T-2 [10]. In the presence of NADPH a mixed function oxidase has been reported to give 3′-hydroxy T-2 and 3′-hydroxy HT-2 in mice liver fractions [11]. Our experiments, without addition of NADPH, would not reveal these metabolites, although we used the same isolation procedure for trichothecenes as used by Yoshizawa *et al.* [11].

Toxicity of HT-2 is found to be comparable to that of T-2. HT-2 is 1.7-fold less toxic than T-2 when administered I.P. [23] to mice. With respect to the rapid metabolism of T-2 to HT-2 by various laboratory animals, it has been suggested that the toxic effects of T-2 toxin is exerted by HT-2 toxin mainly and this metabolic change is not considered to be detoxification of T-2 toxin [13, 15].

Our finding of the increased toxicity of T-2 toxin in TOCP treated animals (2–3-fold) can at least in part be explained by the different toxicity of T-2 toxin and HT-2 toxin.

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