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# Reactivity of 12,13-Epoxytrichothecenes with Epoxide Hydrolase, Glutathione-S-Transferase and Glutathione

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Interaction of the 12,13-epoxytrichothecenes and related mycotoxins with epoxide hydrolase and glutathione-S-transferase (GSH-S-transferase) from rat liver was examined *in vitro*. Neither hydrolysis of safrole oxide by the microsomal epoxide hydrolase nor conjugation of 2,3-epoxy-(*p*-nitrophenoxy)propane with GSH by the soluble GSH-S-transferase was interfered with the trichothecenes such as T-2 toxin and fusarenon-X. Gas-liquid chromatography (GLC) analysis and colorimetric determination of the residual GSH revealed that the trichothecenes were inert to the partially purified GSH-S-transferase. In contrast to the trichothecenes, PR-toxin, an epoxide mycotoxin from *Penicillium roqueforti*, and lactones such as patulin and penicillic acid from *Penicillium* and *Aspergillus* spp., were found to react non-enzymatically with GSH in molar ratio of 1:1.

**Keywords**——12,13-epoxytrichothecene; mycotoxins; T-2 toxin; fusarenon-X; penicillic acid; patulin; PR-toxin; epoxide hydrolase; GSH-S-tarnsferase; glutathione

Mycotoxins encompass a group of toxic small molecules produced by a various species of fungi. Although the injurious effect of mycotoxins on humans and animals has been studied from the standpoint of toxicological and pathological lessions, the basic mechanism of action for most mycotoxins is still poorly understood. Since it is known that many biologically active substances exert their effect after being bound, covalently or non-covalently, to macromolecules and other constituents of organisms, the interaction of mycotoxins with such molecules has attracted a great concern in addition to the other attention such as metabolism and carcinogenecity of mycotoxins.

In the preceding paper, the authors reported that 12,13-epoxytrichothecenes such as T-2 toxin, neosolaniol and fusarenon-X possess the ability to bind with SH-enzymes through active SH-residue of protein molecules.[2)] All the trichothecenes possess an epoxide ring at the C-12, 13, except crotocin possesses an additional epoxide at the C-7,8, and these epoxides are presumed to be an important functional group for exhibiting their cytotoxicity as well as the above mentioned SH reactivity.[3,4)]

Generally speaking, epoxide hydrolase, which is localized in the microsomes of liver, hydrolyses the epoxide ring of many biologically active compounds,[5)] and furthermore, glutathione (GSH)-S-transferase, which is localized in the soluble fraction of liver, catalyzes the enzymatic conjugation between the epoxides and GSH.[6)] These enzyme systems are considered to play an important role in the cellular detoxication of epoxides and other electrophilic compounds.

In this communication, the authors examined the enzymatic and non-enzymatic reactivities of epoxide mycotoxins and other related toxic fungal metabolites with epoxide hydrolase, GSH-S-transferase and GSH. T-2 toxin, fusarenon-X and nivalenol were isolated from the

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metabolites of *Fusarium* spp.[7,8] HT-2 toxin was enzymatically prepared from T-2 toxin.[9] Penicillic acid and patulin were isolated from the culture filtrates of *Penicillium olivino-viride* and *P. expansum*, respectively. Crotocin and PR-toxin were kindly gifted by Drs. E.T. Gláz (Budapest) and R.D. Wei (Taiwan), respectively.

Livers obtained from male Wistar or Sprague-Dawley rats (6 weeks old), which were free from feed overnight before sacrifice, were homogenized with 4 volumes of 0.25 M sucrose, and the homogenates were centrifuged at 9000 ***g*** for 20 min. The resulting S-9 fraction was further centrifuged at 100 000 ***g*** for 90 min, and after washing once with 0.25 M sucrose, microsomes were suspended into 0.15 M KCl at the concentration of 1 g equivalent liver/ml and used for the assay of epoxide hydrolase. The enzyme activity was estimated photometrically according to the method of Watabe, *et al.*[10] with safrole oxide as a substrate. As for the estimation of GSH-S-transferase, the method of Fjellsteld, *et al.*[6] and Kaplowits, *et al.*[11] was essentially adopted with 2,3 epoxy-(*p*-nitrophenoxy)propane (ENP) as a substrate and a 100 000 ***g*** × 90 min supernatant fraction (S-100) as enzyme source. In some experiments, the supernatant fraction was partially purified by precipitating with $(NH_4)_2SO_4$ at 35—70% saturation and the following dialysis of the precipitate against 5% polyethylene glycol. The activity was expressed as $\Delta E_{360}$/mg protein/min.

The amount of GSH was colorimetrically determined with glutathione reductase (Boehringer Mannheim), 5,5′-dithiobis-(2-nitrobenzoic acid) and NADPH.[12] Trichothecenes were quantitatively determined by gas-liquid chromatographic (GLC) technique developed in our laboratory.[9,13]

Table I. Effects of Trichothecenes on Epoxide Hydrolase and GSH–S–Transferase

| Trichothecenes | Concentration (mM) | Epoxide hydrolase[a] (μmol SO hydrolyzed/ mg protein/15 min) | GSH–S–transferase[b] ($\Delta E_{360}$/mg protein) |
|---|---|---|---|
| T-2 toxin | 0 | 0.59 | 0.019 |
| | 2 | 0.52 | |
| | 4 | 0.51 | 0.012 |
| | 8 | 0.52 | |
| | 16 | 0.56 | |
| Fusarenon-X | 0 | 0.63 | 0.018 |
| | 2 | 0.60 | |
| | 4 | 0.56 | 0.018 |
| | 8 | 0.56 | |
| | 16 | 0.55 | |

*a*) 0.08 M phosphate buffer (pH 7.4), 0.4 mM safrol oxide, 0.5 mM microsomes (40—50 mg equivalent liver), in total volume of 2.55 ml, were incubated at 37° for 15 min in the presence or absence of trichothecenes.
*b*) 0.1 M phosphate buffer (pH 6.5), 10 mM GSH, 0.5 mM ENP and 0.2 ml S-100 (2 mg protein), in total volume of 3.0 ml, were incubated at 37° in the presence or absence of trichothecenes. The increase of absorption at 360 nm was estimated with an interval of 1 min.

With an aim to demonstrate the possible interaction of trichothecenes with microsomal epoxide hydrolase and cytosol GSH-S-transferase of rat liver, the effect of T-2 toxin and fusarenon-X on these two enzyme activities was examined. As summarized in Table I,

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neither T-2 toxin nor fusarenon-X interfered directly with the enzymatic hydrolysis of SO by the microsomes at the concentration up to 16 mM, which was 40 times higher than that of the substrate. These two mycotoxins also did not interfere with the S-100 catalyzed conjugation of ENP and GSH at the concentration of 4 mM, 8-fold higher than the substrate. Even when these enzymes were preincubated with the trichothecenes in the absence of respective substrates, the following enzymatic reactions proceeded without any inhibition. These data indicated that the trichothecenes failed to compete with SO or ENP to respective enzymes, or these enzymes did not attack the epoxide of trichothecenes.

In order to obtain the direct evidence that the trichothecene compounds are inert to the GSH-S-transferase, the GLC determination of trichothecenes was performed. The partially purified transferase was incubated with T-2 toxin and fusarenon-X in the presence or absence of added GSH, and the trichothecenes in the reaction assay system were determined by GLC. As summarized in Table II, the liver extract produced HT-2 toxin and nivalenol from T-2 toxin and fusarenon-X, respectively, in agreement with the previous finding that the liver enzyme(s) hydrolysed selectively the acetyl group at C-4 of trichothecenes.[9] However, the total amount of residual substrates (T-2 toxin and fusarenon-X) and the newly produced metabolites (HT-2 toxin and nivalenol) is almost the same as that of added substrates either in the presence or absence of GSH. These data indicate that the liver extract catalyzes only the deacetylation reaction of trichothecenes and that the transfer of trichothecenes to GSH does not take place. Low recovery of the total trichothesenes in case of fusarenon-X (Table II-b) may be caused by a low recovery of nivalenol under ethylacetate extraction.

Table II. GLC Analysis of Trichothecenes in GSH–S–Transferase Reaction System

a) T-2 toxin as Substrate

| GSH (20 mM) | Incubation (37° 180 min) | Trichothecenes (μmol/assay) | | |
|---|---|---|---|---|
| | | T-2 toxin | HT-2 toxin | Total |
| + | − | 1.01 (100)[a] | 0 | 1.01 (100) |
| − | + | 0.42 ( 42) | 0.57 (56) | 0.98 ( 98) |
| + | + | 0.61 ( 62) | 0.42 (42) | 1.05 (104) |

b) Fusarenon-X as Substrate

| GSH | Incubation | Fusarenon-X | Nivalenol | Total |
|---|---|---|---|---|
| + | − | 1.33 (100) | 0 | 1.33 (100) |
| − | + | 0.60 ( 46) | 0.25 (19) | 0.85 ( 65) |
| + | + | 0.74 ( 55) | 0.14 (10) | 0.88 ( 67) |

*a*) Parenthesis indicates the percent of control.
A mixture (1.01 ml) containing 100 μmol of phosphate buffer (pH 6.5), 20 μmol of GSH, 1 μmol of T-2 toxin or fusarenon-X and 0.8 ml of partially purified transferase was incubated at 37° for 0 or 180 min, and the trichothecenes were three times extracted by chloroform (T-2 toxin and HT-2 toxin) or ethylacetate (fusarenon-X and nivalenol).

Another approach was made with a colorimetric determination of residual GSH in the reaction system of GSH-S-transferase, as summarized in Table III. With ENP as substrate, a half of GSH added was consumed. On the other hand, with the trichothecenes such as T-2 toxin, HT-2 toxin, fusarenon-X and crotocin, no significant consumption of GSH was observed.

From these evidences, the epoxide of trichothecenes is hardly reactive with GSH-S-transferase of rat liver. This finding is contract with Foster, *et al.*[14] who reported that the trans-

14) P.M.D. Foster, T.F. Slater, and D.S.P. Patterson, *Biochem. Soc. Trans.*, 3, 875 (1975).

ferase catalyses the formation of GSH-conjugate of trichothecenes such as T-2 toxin and diacetoxyscirpenol.

Since the epoxide of trichothecenes are found to be inert to GSH and GSH-S-transferase, the authors examined the reactivity of other kinds of epoxide and lactones, which exhibit

Table III. Residual GSH in GSH-epoxide Transferase Reaction

| GSH (5 mM) | Incubation | | Substrates (5 mM) | Residual GSH (μg/assay) | Residual GSH (%) |
|---|---|---|---|---|---|
| + | 37° | + | — | 1339 | 88 |
| + | | + | ENP | 694 | 42 |
| + | | + | T-2 toxin | 1519 | 99 |
| + | | + | Fusarenon-X | 1384 | 90 |
| + | | + | HT-2 toxin | 1520 | 99 |
| + | 25° | + | — | 1471 | 96 |
| + | | − | ENP | 1466 | 95 |
| + | | − | Crotocin | 1606 | 104 |
| + | | + | ENP | 1171 | 76 |
| + | | + | Crotocin | 1606 | 105 |

0.1 M phosphate buffer (pH 6.5), 5 mM GSH (1535 μg) and 0.2 ml (10 mg protein) S-100, in total volume of 1.0 ml, were incubated at 25° or 37° in the presence or absence of substrates.

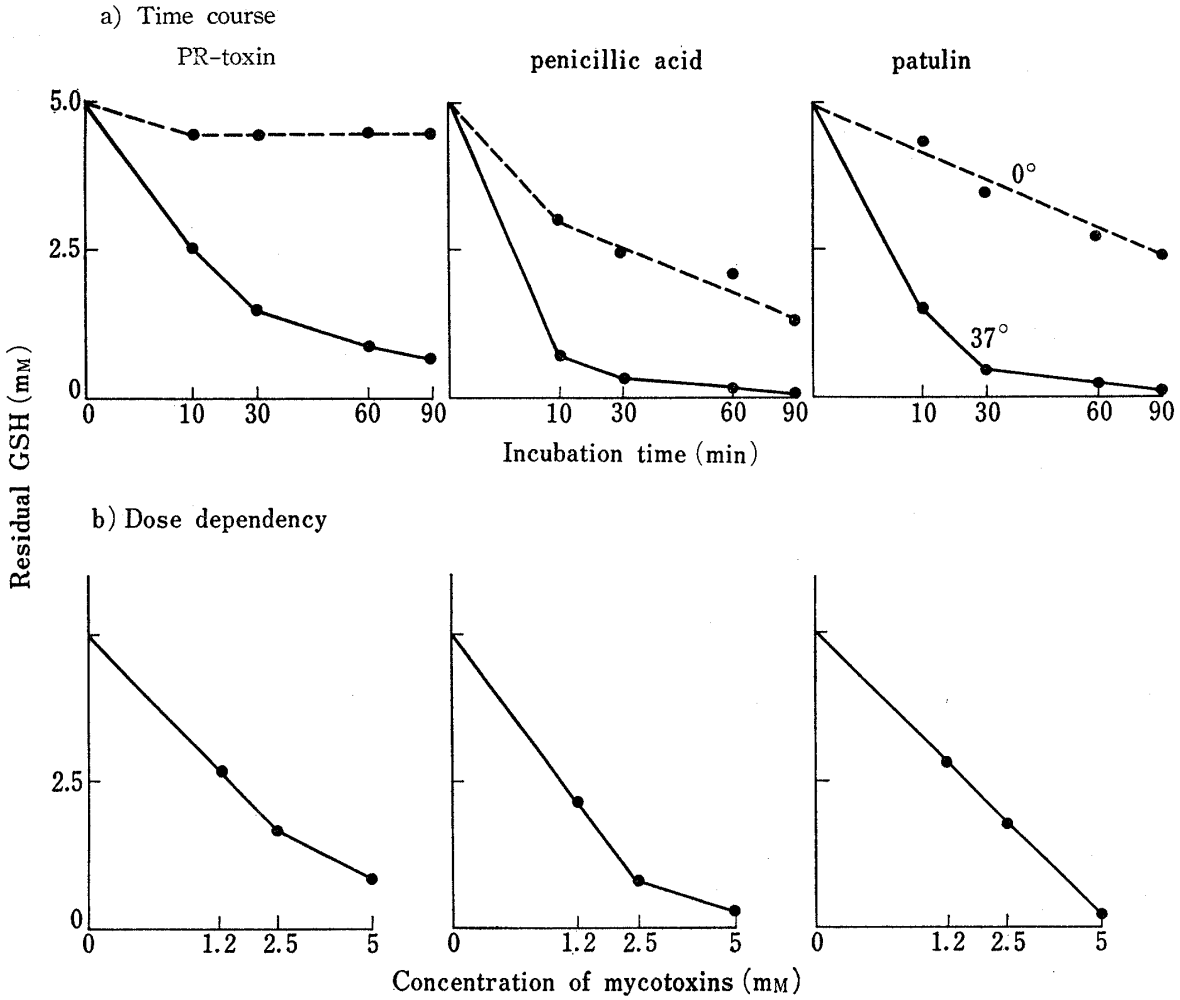


Fig. 1. Non-enzymatic Reactivity of PR-toxin, Patulin and Penicillic Acid with GSH

A mixture (0.4 ml) containing 0.1 M phosphate buffer (pH 6.5), 5 mM GSH and 5 mM mycotoxin was incubated at 0° or 37° for 90 min (a). In Exp. (b), GSH content was fixed to 5 mM and each mycotoxin was varied from 1.25 mM to 5 mM, and the mixture was incubated at 37° for 60 min.

toxicity to animal tissues. After incubation of a mixture (0.4 ml) containing 0.1 M phosphate buffer (pH 6.5), 5 mM GSH and 5 mM test compounds at 0° or 37° for 90 min, the residual GSH was assayed. With PR-toxin, patulin and penicillic acid, the residual GSH was gradually decreased in parallel to the time of incubation, and this non-enzymatic conjugation proceeded more rapidly at 37° than at 0°, as shown in Fig. 1-a. Dose–response curves demonstrated that total GSH (5 mM) was completely consumed at 5 mM of each substrate (Fig. 1-b). Therefore, it is presumed that these mycotoxins are able to react with GSH in molar ratio of 1:1. As for patulin and penicillic acid, the SH-reactivity was proposed.[15] As for PR-toxin, the additional experiment has revealed the reactivity with cystein, dithiothreitol and CoA.

Summing up the above experiments, it is concluded that the epoxide of trichothecenes is inert to epoxide hydrolase, GSH-S-transferase and GSH. On the other hand, PR-toxin, patulin and penicillic acid react non-enzymatically with GSH. Difference in SH-reactivity between the epoxide of trichothecenes and that of PR-toxin remains to be solved.

Oesch, *et al.*[16] investigated the structure–activity relationships for substrates and inhibitors of hepatic epoxide hydrolase. Oxiranes with a 1-aryl substituents or with certain 1-alkyl substituents are proved to be the best substrates and di-, tri-, and tetrasubstituted oxiranes are virtually inactive. From these stereospecificity of epoxide hydrolase, it is conceivable that 12,13-epoxytrichothecenes are inactive as a substrate or inhibitor of epoxide hydrolase.

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