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Inactivation of Some Thiol-Enzymes by Trichothecene Mycotoxins from *Fusarium* Species

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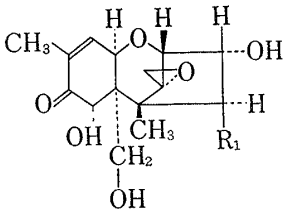
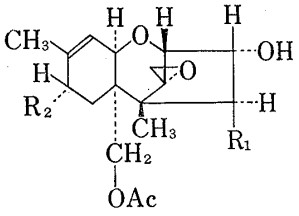
Fusarenon-X, neosolaniol and T-2 toxin, 12,13-epoxytrichothecene mycotoxins of *Fusarium* spp. cause *in vitro* an inactivation of SH-enzymes such as creatine phosphokinase, lactate dehydrogenase and alcohol dehydrogenase when the enzyme molecules were preincubated with the mycotoxins in the absence of substrates, and the supplement of dithiothreitol prevents this inactivation. Gel-filtration of a mixture containing alcohol dehydrogenase and ^{3}H-labelled fusarenon-X revealed the formation of a complex [^{3}H-fusarenon-X-alcohol dehydrogenase] with a molecular ratio of 4: 1, and dithiothreitol prevented the complex formation. These results indicate that the epoxytrichothecenes bind with the thiol residues of SH-enzyme protein.

The 12,13-epoxytrichothecene mycotoxins (Fig. 1) produced by the fungi *Fusarium* species[2,3,4] are highly toxic to proliferating eukaryotic cells.[5] Our studies have indicated that the trichothecenes are a potent and specific inhibitor of protein synthesis on mice tissues,[6] rabbit reticulocytes,[7,8,9] protozoa[10] and Ehrlich tumor cells.[6,9,11]



	R_1
fusarenon-X:	OAc
nivalenol:	OH



	R_1	R_2
T-2 toxin:	OAc	$(CH_3)_2CHCH_2OCO$
HT-2 toxin:	OH	$(CH_3)_2CHCH_2OCO$
neosolaniol:	OAc	OH

Fig. 1. 12,13-Epoxytrichothecenes

In order to elucidate the mechanism of the inhibitory action, the binding capacity *in vitro* of the mycotoxins with ribosomes or polysome of rat liver and rabbit reticulocytes was assayed.

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The results indicated that fusarenon-X and T-2 toxin bind to the polysome to cause an impairment of the peptidyl transferase center which is integrated within the ribosomes and that the treatment of the ^{3}H-trichothecene-bound polysomes with detergents such as deoxycholate eliminated the bound mycotoxins from the particle.[12] From these findings it is presumed that the toxins are associated with the protein moiety of ribosomes.

As a step to clarify the binding mode of the trichothecenes with protein, we examined an inhibitory effect of the trichothecene compound on SH-enzymes such as creatine phosphokinase [EC. 2.7.3.2] from rabbit muscle, alcohol dehydrogenase [EC. 1.1.1.1] from yeast and lactate dehydrogenase [EC. 1.1.1.27] from rabbit muscle, because it has been established that all the toxic trichothecenes possess an epoxide ring which is presumed to be an important functional group for exhibiting their cytotoxicity[4] and that thiols add spontaneously their SH groups to epoxides.[13]

Fusarenon-X, T-2 toxin and neosolaniol were isolated from the culture filtrates of *Fusarium* spp.[3,14,15] ^{3}H-fusarenon-X (2.9 mC/mM) labelled by Wilzbach's method, was purified by silicagel column chromatography followed recrystallization from dichloromethane–*n*-pentane. All the enzymes were the product of Boehringer Mannheim and the enzymatic activities of creatine phosphokinase,[16] alcohol dehydrogenase[17] and lactate dehydrogenase[18] were determined by measuring a change of absorbancy of NAD at 340 nm according to the methods reported elsewhere.

Table I. Inhibition of Alcohol Dehydrogenase by Fusarenon-X

Fusarenon-X (mM)	DTT (mM)	Preincubation time (min)	NADH (O.D./min)	Activity (%)
0	—	—	0.150	100
3	—	—	0.150	100
10	—	—	0.150	100
0	—	5	0.070	100
0.5	—	5	0.070	100
5	—	5	0.040	57
10	—	5	0	0
0	—	15	0.050	100
0.6	—	15	0.010	20
5	—	15	0	0
0	10	5	0.070	100
0.6	10	5	0.070	100
5	10	5	0.070	100
10	10	5	0.070	100
0	10	15	0.050	100
0.6	10	15	0.050	100
5	10	15	0.050	100
10	10	15	0.050	100

90 μmoles of Na-pyrophosphate buffer (pH 8.5), 900 μmoles of ethanol, and 0.45 μmole of NAD in total volume of 2.85 ml, were incubated at 25°, and the reaction was started by addition of 0.15 ml of the enzyme solution which consisted of 10 μg of alcohol dehydrogenase and 10 μmoles of the buffer. In some experiments, the enzyme solution was preincubated with fusarenon-X at 25° for 5 or 15 minutes in the presence or absence of dithiothreitol (DTT).

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The trichothecenes such as fusarenon-X, neosolaniol and T-2 toxin caused no inhibitory effect on the enzyme activity of creatine phosphokinase in concentrations up to 10 mM when the three kinds of mycotoxins were added directly to the reaction mixture containing the substrate and the enzyme. Whereas, when the enzyme without the substrate was preincubated with 30 mM of the mycotoxins at 25° for 10 minutes the activity was markedly reduced and the complete inactivation was attained at 10 mM of the mycotoxin. Similar results were obtained with fusarenon-X and lactate dehydrogenase from rabbit muscle, in which the complete inactivation was resulted with 50 mM of the mycotoxin.

Further experiments with alcohol dehydrogenase from yeast were summerized in Table I. The enzyme was not inhibited by 3 and 10 mM of fusarenon-X when assayed without preincubation. Whereas, the activity was significantly reduced when preincabated with 5 mM and 0.6 mM of fusarenon-X for 5 min and 15 min, respectively, and these inactivations were prevented by the preincubation in the presence of dithiothreitol.

In order to exclude the possibility that the inhibitory effect of fusarenon-X is diminished by reacting with SH-group of dithiothreitol, a mixture containing 5 mM of fusarenon-X and 10 mM of dithiothreitol was preincubated at 25° for 30 minutes followed by addition to the

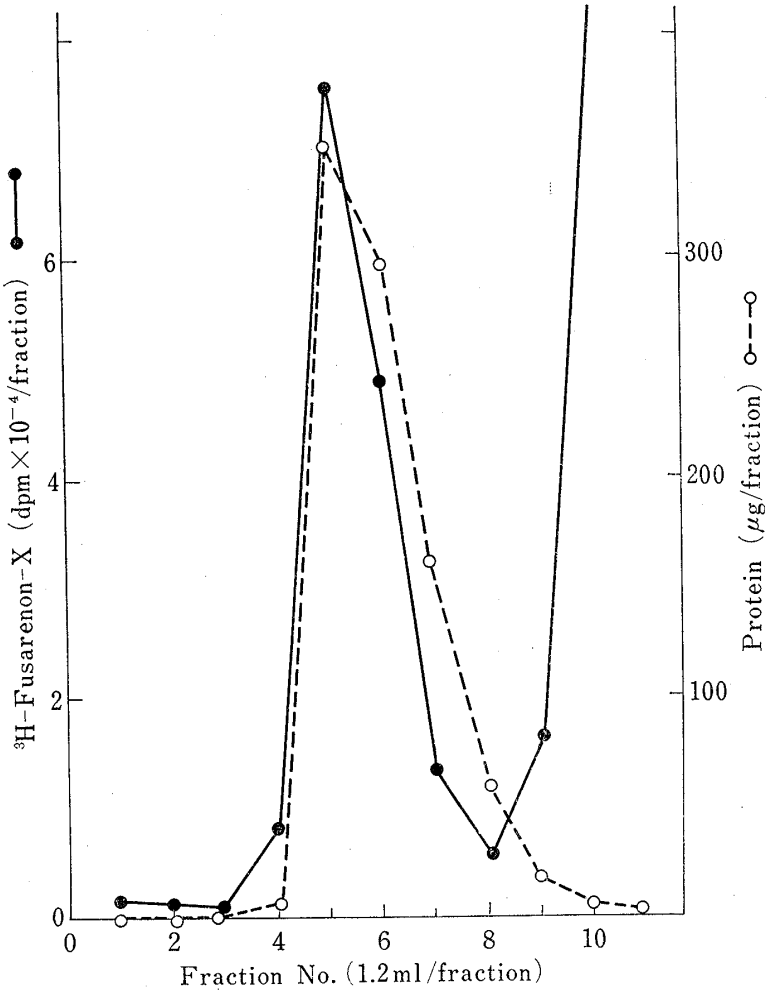

Fig. 2. Binding Reaction of ^{3}H-Fusarenon-X to Alcohol Dehydrogenase

A mixture (1 ml) containing 200 μg (0.6 μmole) of ^{3}H-fusarenon-X-(2.9 mc/mM), 1.5 mg (0.01 μmole) of alcohol dehydrogenase (270000 units/mg) and 5 μmoles of phosphate buffer (pH 7.4) was incubated at 28° for 15 minutes and followed by gel-filtration on Sephadex G75 column (1×25 cm) with 0.01M phosphate buffer (pH 7.4) at 4° with a flow rate of 1.2 ml/fraction/5 minutes.

enzyme assay system. No reduction of the inhibitory potency of fusarenon-X was observed. Furthermore, a lethal toxicity test to mice (5) and reticulocyte bioassay (7) demonstrated that the toxicity of fusarenon-X solution containing an equimolar amount of dithiothreitol, cysteine or glutathione was the same as that of fusarenon-X alone. From these findings, it is very likely that fusarenon-X does not react with SH-group of dithiothreitol and other SH-compounds under the experimental condition.

From the above findings and the data presented in Table I, the trichothec mycotoxins were presumed to react with SH-groups of the active center of the enzyme molecules.

With an aim to clarify the possibility that the trichothecenes bind to the enzyme, 0.01 μmole of alcohol dehydrogenase was preincubated at 28° for 15 minutes with 0.6 μmole of ^{3}H-fusarenon-X and was gel-filtrated over Sephadex G-75, and distributions of the protein and fusarenon-X were monitored by measuring the optical density at 260 nm and the radioactivity, respectively. As shown in Fig. 2, the ^{3}H-fusarenon-X found in protein fractions was separated from the free fusarenon-X fraction. No measurable counts of fusarenon-X was detected in the protein fraction when the enzyme was preincubated as above in the presence of 10 μmoles of dithiothreitol. Assuming the molecular weight of yeast alcohol dehydrogenase to be 148000,[19] the two separate experiments demonstrated that one mole of the enzyme molecule binds with 3.6 and 3.4 moles of fusarenon-X.

According to Whitehead, *et al.*[20] and Jocelyn,[21] alcohol dehydrogenase possess totally 36 SH-residues among which four SH-residues are highly reactive on SH-reagents. From these properties of the enzyme protein and the experimental data on the inactivation and the binding reaction of SH-enzymes with trichothecenes, as described above, it may be concluded that the 12,13-epoxytrichothecene mycotoxins are capable to inactivate the SH-enzymes through binding with reactive SH-residues of the active center of the enzyme molecule. The role of this biochemical feature of the toxic trichothecenes in an inhibitory mechanism of protein synthesis is under investigation.

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