NOTES

IN VITRO INHIBITION OF BOVINE LIVER GLUTAMATE DEHYDROGENASE BY CITRININ, A MYCOTOXIN

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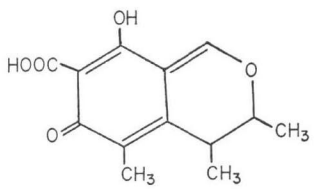
(Received for publication November 16, 1976)

Citrinin, a hepato-nephrotoxic principle, is produced by fungi belonging to the genera *Aspergillus* and *Penicillium*[1]. Some of these fungi commonly contaminate food materials and elaborate citrinin[2,3] and thus pose a serious health hazard.

Studies on the effect of this antibiotic on rabbit liver metabolism indicated that there is increased synthesis of lipids[4]. However, ENDO and KURODA[5] have recently shown inhibitory effect of citrinin on cholesterol and triglycerides biosynthesis in rat liver. Preliminary studies on isolated rabbit liver mitochondria from citrinin-treated animals indicate that there is decreased rate of oxidation of substrates as compared to the control mitochondria[3]. Also, our recent studies indicate uncoupling of oxidative phosphorylation in dog heart mitochondria by citrinin. In the light of these observations it was thought worthwhile to check the effect of citrinin on some of the key enzymes like phosphofructokinase (E.C. 2.7.1.56), citrate synthase (E.C. 4.1.3.7) and glutamate dehydrogenase (E.C. 1.4.1.3) *in vitro*.

Spectroscopically pure crystalline citrinin was prepared as described previously[6]. Phosphofructokinase was prepared according to the procedure of LING *et al.*[7] with slight modifications[8] from rabbit muscle and assayed as described by SOLS[9]. Citrate synthase was purified from rat liver according to the method of MUKHERJEE and SRERE[10] and the activity determined by measuring the rate of CoASH formation in the presence of DTNB[11]. Bovine liver glutamate dehydrogenase (obtained from Boehringer) activity was measured by following the oxidation of NADH at 340 nm[12].



Abbreviations used: DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); F6P, fructose-6-phosphate.

When citrate synthase, or glutamate dehydrogenase was preincubated with citrinin (1 mM) at 35°C for 15 minutes, and then assayed, only glutamate dehydrogenase showed a 60% loss of activity. Citrate synthase activity on the other hand remained unaffected. When phosphofructokinase was incubated with citrinin (1 mM) and assayed under optimal conditions (pH 8.0 assay in the presence of 1 mM F6P, 1 mM ATP and 5 mM $(NH_4)_2SO_4$), there was no change in the activity observed (Table 1). To rule out the possibility of citrinin binding to phosphofructokinase affecting its allosteric properties, the assay was carried out at pH 7.0 with 0.1 mM F6P,

Table 1. Effect of citrinin on the activity of citrate synthase, glutamate dehydrogenase and phosphofructokinase.

Addition	Citrate synthase ΔOD/min 412 nm	Glutamate dehydrogenase ΔOD/min 340 nm	Phosphofructokinase ΔOD/min 340 nm
Control	0.15	0.075	0.10
Citrinin	0.16	0.030	0.10

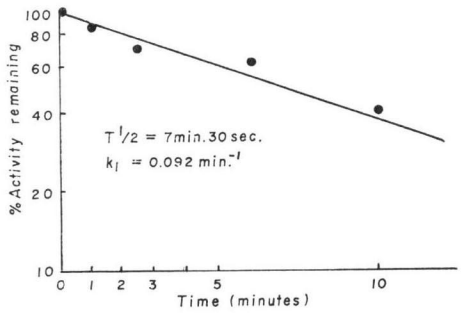
Citrinin was solubilized in ethanol to give 0.1 M solution. To the control incubations 10 μl of ethanol was added. Citrate synthase (65 μg/ml) in 0.01 M tris-HCl pH 7.5 was incubated with citrinin at 35°C for 15 minutes. Thereafter it was diluted 1: 4 times in 0.01 M tris-HCl pH 7.5 and aliquot was used for assay.

Glutamate dehydrogenase (62.5 μg/ml) in 0.01 M tris-HCl pH 7.5 was incubated with citrinin (1 mM) at 35°C for 15 minutes and then an aliquot was used for assay.

Phosphofructokinase (70 μg/ml) in 0.05 M tris-PO_4 buffer pH 8.0 containing 0.2 mM EDTA was incubated with citrinin (1 mM) at 35°C for 15 minutes and then diluted 1: 10 in 0.05 M tris-PO_4 buffer pH 8.0. Aliquot was used for assay.

Fig. 1. Time course of inhibition of bovine liver L-glutamate dehydrogenase by citrinin.

Citrinin (1 mM) was incubated with the enzyme (62.5 μg/ml) at 35°C and at varying time intervals an aliquot was taken and assayed for the enzyme activity. Controls were run concurrently with the enzyme incubated alone at 35°C.



1 mM ATP, 5 mM NH_4Cl, and 2 mM $MgCl_2$. Even under these conditions there was no activity difference between the controls and the citrinin-treated enzyme. It must be pointed out here that the possibility of an *in vivo* modified product of citrinin interacting with phosphofructokinase or citrate synthase cannot be ruled out.

The time course of inhibition of glutamate dehydrogenase by citrinin appeared to indicate a first order reaction with a $t\frac{1}{2}$ of 7 $\frac{1}{2}$ minutes and the rate constant (k) was calculated to be 0.092 min^{-1} (Fig. 1).

With increasing concentration of citrinin, there was increasing extent of inhibition, until a maximum inhibition of about 60% was achieved with 1 mM citrinin (Fig. 2). Further increase in citrinin concentration, did not produce any further inhibition. This may be due to the fact that citrinin being sparingly soluble in water has reached maximum solubility at 1 mM concentration under incubation conditions.

When α-ketoglutarate was added prior to the addition of citrinin and preincubated, the substrate offered protection against citrinin inhibition. However, this was dependent upon concentration of the substrate and complete protection was observed at a concentration of 3.2 mM (Fig. 3).

Since ADP is an activator of glutamate dehydrogenase[13], the effect of this nucleotide on citrinin inhibition was tested. As shown in Table 2, preincubation with ADP (up to a concentration of 500 μM) increased the activity of

Fig. 2. Effect of increasing concentration of citrinin on bovine liver L-glutamate dehydrogenase.

Varying concentration of citrinin was incubated with the enzyme (62.5 μg/ml) at 35°C and after 15 minutes an aliquot was taken for the enzyme activity.

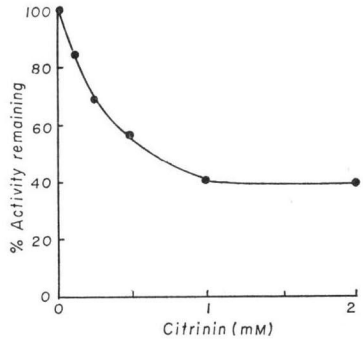
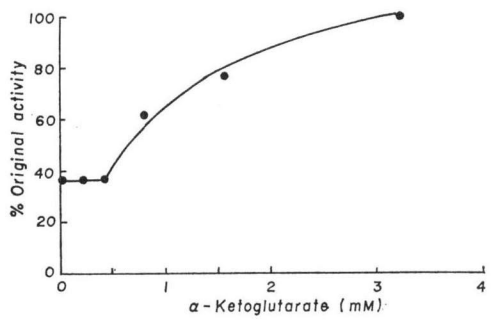


Fig. 3. Protective action of α-ketoglutarate against the inhibition of bovine liver L-glutamate dehydrogenase (62.5 μg/ml) by citrinin (1 mM).

Varying concentration of α-ketoglutarate was present during the enzyme incubation with citrinin at 35° C. An aliquot was assayed after 15 minutes of incubation for enzyme activity.



the enzyme by about 2.5 fold. In the presence of citrinin, only 25 ~ 30% of the activity remained, suggesting that ADP does not protect the enzyme from inhibition. However, ADP stimulated the activity of the uninhibited enzyme. When the enzyme was assayed with NADPH, a co-factor which has been shown to be preferentially utilized by glutamate dehydrogenase in the mitochondria[14,15] a similar inhibition pattern was observed.

Of the regulatory enzymes tested only glutamate dehydrogenase was irreversibly inhibited by citrinin. Mycotoxin luteoskyrin produced by *Penicillium islandicum* has been shown to

Table 2. Effect of ADP on citrinin inhibition of glutamate dehydrogenase.

Additions	Assay with NADH		Assay with NADPH	
	⊿OD/min 340 nm	% inhibition	⊿OD/min 340 nm	% inhibition
Control	0.075	—	0.062	—
Citrinin (1 mM)	0.023	69	0.0165	74
ADP (100 μM)	0.138	—	0.145	—
ADP (100 μM)+ Citrinin (1 mM)	0.042	70	0.04	72
ADP (200 μM)	0.150	—	—	—
ADP (200 μM) + Citrinin (1 mM)	0.055	63	—	—
ADP (500 μM)	0.190	—	0.146	—
ADP (500 μM) + Citrinin (1 mM)	0.048	75	0.038	74

Conditions of incubation are as given under Table 1.
ADP was neutralized to pH 7.0 prior to use.

inhibit succinate dehydrogenase, D-amino acid oxidase and cytochrome C oxidase activity *in vitro* (see ref. 2). Similarly, kojic acid was reported to inhibit D-amino acid oxidase activity *in vitro*. The nature of inhibition was however, shown to be of the competitive type[16].

The possibility that citrinin interaction results in depolymerization of the enzyme and the implication of such an effect on liver metabolism are being looked into.

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