Inhibitory Actions of Mycotoxins on Brain γ-Aminobutyrate Transaminase

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GABA transaminase (4-aminobutyrate aminotransferase), which catalyzes the breakdown of the major inhibitory neurotransmitter, GABA, in mammalian brain, was inactivated by preincubation with the mycotoxin patulin. The time course of the reaction was significantly affected by the substrate α-ketoglutarate, which afforded complete protection against the loss of catalytic activity. The recovery from the inhibition of patulin by the addition of dithiothreitol (DTT) supports that patulin reacts with the sulfhydryl residue in the catalytic domain of the enzyme. The reconstitution of the reduced enzyme and apoenzyme with pyridoxal-5-P(PLP) was inhibited by another mycotoxin, penicillic acid. This mycotoxin may interact with lysyl residue of the enzyme. Therefore, it is postulated that the critical sulfhydryl and lysyl residues in the catalytic domain of the enzyme react with mycotoxin patulin and penicillic acid, respectively.

KEY WORDS □ GABA transaminase, neurotransmitter, mycotoxin, patulin, penicillic acid.

Patulin(4-hydroxy-4-H-furo[3,2-c]pyran-2(6H)-one) and penicillic acid are fungal toxins with an α,β unsaturated lactone structure produced by several species of *Aspergillus* and *Penicillium* (1). Both toxins exhibit antibiotic activity and can be potential health hazards as contaminants of agricultural products, especially apples, apple products, and cider (24).

Moreover, patulin is toxic to numerous biological systems. Pathologies include severe hemorrhage and capillary damage in liver, spleen and kidney along with edema of the brain in rats and nervous symptoms, cerebral hemorrhage and death in mice (28). It has been also reported that patulin has mutagenic and carcinogenic properties (19, 27). The number of different kinds of proteins and enzymes in *Escherichia coli* may be inactivated by different concentrations of agents like patulin reacting with SH groups (14). Various enzymes with essential thiol groups, such as lactate dehydrogenase (2), muscle aldolase (3), and RNA polymerase (25) can be inhibited by patulin. Although the toxic effects of mycotoxins on animals have been studied from pathological, toxicological and biochemical standpoints, the basic mechanisms of these mycotoxins are still poorly understood.

*This work was supported by a grant from Hallym University to S.Y. Choi in 1991.

GABA(γ-aminobutyrate) has been shown to be an important inhibitory neurotransmitter (21). The concentration of GABA in the brain is regulated principally by the enzymes that biosynthesize it, L-glutamate decarboxylase, and the enzyme that degrades it, GABA transaminase (4-aminobutyrate aminotransferase: EC 2.6.1.19). When the brain GABA level diminishes to below a threshold level, various neurological disorders, including seizures, convulsions, Huntington's disease (23), epilepsy (26), and Parkinsonism (20), may occur.

It has been reported that the purified GABA transaminase from pig brain is a dimer made up of two identical subunits of M.W. 50,000 (15) and possesses two nonequivalent catalytic binding sites differing in their affinities for the cofactor PLP (9) which interacts with ε-lysine residues of the enzyme. The behavior of the enzyme is very unusual in the sense that the K_{cat} and K_m values of the enzyme containing 1 mole PLP/dimer are identical with those of the enzyme containing 2 mole PLP/dimer. A second catalytic site on the dimeric enzyme becomes functional only after specific chemical modification of the molecule of cofactor tightly bound to the enzyme (10). There is strong evidence that lysyl and cysteinyl residues are connected with the catalytic activity of GABA transaminase (7, 15). Therefore, it is main purpose of this work to investigate the inhibitory action of these mycotoxins on GABA transaminase and

to probe the amino acid residues modified by these mycotoxins.

MATERIALS AND METHODS

Materials

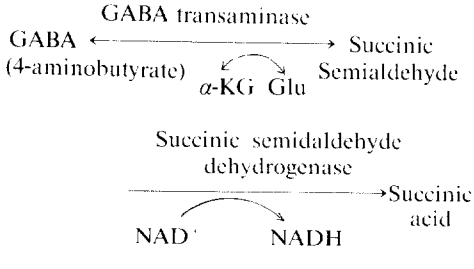
Pig brains were obtained from Majangdong Packing Company in Seoul, Korea. Penicillic acid, succinic semialdehyde, NAD^+, pyridoxal-5-P, α-ketoglutarate were purchased from Sigma. CM-Sephadex, DEAE-Sephadex were purchased from Pharmacia Fine Chemicals. All other chemicals used were analytical grade.

Purification of enzymes

GABA transaminase (GABA-T) was purified from pig brain according to a procedure previously described (17). This prepared enzyme had a specific activity of 20 units/mg at 25°C and migrated as a single protein on polyacrylamide gel electrophoresis. The enzyme succinic semialdehyde dehydrogenase was purified from pig brain by a method already described (4). Protein concentration was determined by the colorimetric method of Bradford (5).

Enzymatic assays

A coupled assay system (Scheme I) consisting of two purified enzymes, which are GABA transaminase and succinic semialdehyde dehydrogenase, were used to study the catalytic conversion of GABA to succinic semialdehyde. Reduction of NAD^+ to NADH was measured by following the increase in absorbance at 340 nm with standard assay mixture containing GABA (30 mM), α-ketoglutarate (30 mM), NAD^+ (4 mM) and excess succinic semialdehyde dehydrogenase in 0.1 M sodium pyrophosphate buffer, pH 8.4 at 37°C using a concentration of enzyme of 30 μg/ml. A unit of enzyme activity is defined as that amount of enzyme which produces 1 μmol/min of NADH at 37°C.



Preparation of patulin

The preparation of patulin was performed by the method of Norstadt and McCalla (22) except that instead of potato dextrose medium (Difco Laboratory). Commercial apple juice was used and inoculated with 1.2×10^5 spores of *Penicillium patulum* NRRL 5259 per ml. After cell culture at room temperature, patulin was extracted with ethyl acetate from the apple juice culture, and alumina columns (2×15 cm, pH 4.5) were used for purification. Patulin was eluted with and crystallized from ethyl ether.

Purity was tested by thin layer chromatography and detected by reaction with 3-methyl-2-benzothiazolinone hydrazone (MBTH) reagent (24).

Polyacrylamide gel electrophoresis

Discontinuous polyacrylamide gel electrophoresis was performed according to the procedure of Davis (12). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out at 25°C as described by Laemmli (18).

Resolution of holoenzyme

The GABA transaminase (20 μM) in 0.1 M potassium phosphate (pH 7.0) containing 1 mM 2-mercaptoethanol was allowed to react with 40 mM GABA at 25°C for 10 min. The pH of the solution was decreased to pH 6 by addition of KH_2PO_4 to a final concentration of 0.5 M and kept at 4°C for 30 min. Then it was dialyzed against 1 M phosphate monobasic containing 1 mM 2-mercaptoethanol for 1 h at 4°C, followed by dialysis against 0.1 M potassium phosphate (pH 7.0) or 0.05 M triethanolamine HCl buffer (pH 7.0) at 4°C for 3h. The resulting apoenzyme is inactive but regains more than 95% of the original enzymatic activity after addition of pyridoxal-5-P (8).

Reduction of holoenzyme with $NaBH_4$

The GABA transaminase was reduced with excess $NaBH_4$ (3 mg/ml) at 4°C and then dialyzed against 10 mM potassium phosphate buffer (pH 7.0)/1 mM 2-mercaptoethanol. After dialysis, reduced enzyme is enzymatically inactive. However, enzymatic activity is restored to the same level as holoenzyme after preincubation with pyridoxal-5-P (8).

Spectroscopy

Spectrophotometric measurements were carried out in Kontron UVIKON 930 Model double beam spectrophotometer.

RESULTS

Inactivation of GABA transaminase by patulin

The effect of patulin on the catalytic activity of the GABA transaminase was investigated by preincubating the enzyme (10 μM) with about 50 fold molar excess of patulin (5×10^{-4} M) at 25°C in 0.1 M potassium phosphate buffer (pH 7.0). As shown in Table 1, inactivation of the enzyme was attained when the protein was preincubated with the excess of patulin at pH 7.0 for 60 min. Complete protection of the enzyme activity against the inhibitory effect of the patulin was observed by preincubating the enzyme with 10 mM α-ketoglutarate prior to the addition of patulin. However, another mycotoxin penicillic

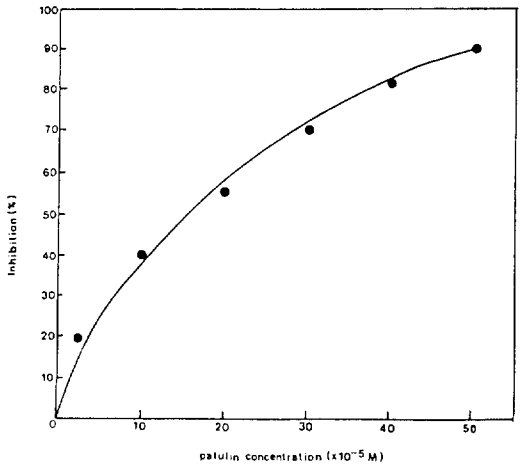


Fig. 1. *Inactivation of GABA transaminase by mycotoxin patulin.*
Enzyme (10 μM) was incubated with various concentrations of patulin at 25°C, pH 7.0.

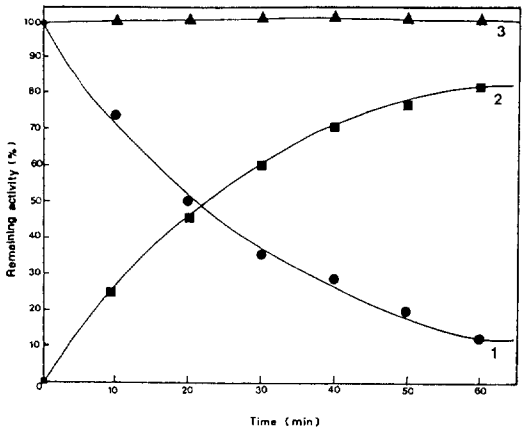


Fig. 2. *Reaction of patulin (500 μM) with GABA transaminase (10 μM).*
Enzyme (10 μM) was incubated with 50-fold molar excess of patulin (500 μM) in the absence (1, ●) and presence (3, ▲) of α-ketoglutarate (5 mM) at 25°C, pH 7.0. Restoration of catalytic activity was done upon addition of dithiothreitol (10 mM) to the inhibited enzyme (2, ■).

acid shows small inhibitory effect on the enzyme activity.

Chemical modification of sulfhydryl residues connected with catalytic activity

The reaction of GABA transaminase (10 μM) with various concentrations of patulin was measured in 0.1 M phosphate buffer (pH 7.0) at

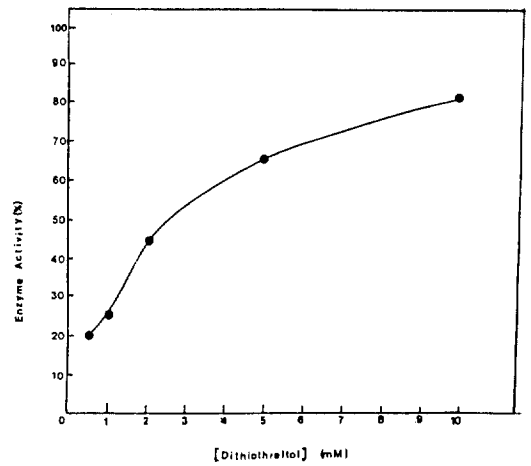


Fig. 3. *Effect of different concentrations of DTT on the inhibition of GABA transaminase (10 μM) by patulin (500 μM).*

25°C (Fig. 1). The time courses for a typical reaction between the enzyme (10 μM) and patulin (500 μM) is given in Fig. 2. The enzyme treated with patulin exhibited 15% of the normal catalytic activity, but the addition of molar excess of dithiothreitol (DTT) with respect to protein rapidly restored 85% of normal catalytic activity. The inhibitory effect exerted by patulin was completely prevented by the presence of the substrate α-ketoglutarate (5 mM) in the reaction mixture when preincubated with GABA transaminase prior to the addition of patulin. To clarify the mechanism of inhibition of GABA transaminase by patulin, a further experiment was made by using the reducing agent, dithiothreitol (DTT). The recovery from the inhibition of patulin by the addition of different concentrations of DTT supports that patulin acts as an -SH blocker (Fig. 3). The fact that patulin is readily inactivated by thiols (11, 13) has led to the hypothesis that the mode of action of this mycotoxin is due to their interaction with the -SH residues in enzyme. The changes of absorption spectra were observed in the reaction mixture of free cystein (1 mM) and patulin (500 μM) as a function of time (Fig. 4). After incubation of free cystein and patulin, the absorption peak immediately moved to the longer wavelength (red shift) and decreased the absorbance at the 314 nm concomitant with small increase at the 256 nm. This spectroscopic changes suggest that the chemical reactions take place between the cystein and patulin. However we couldn't detect large spectral changes of the enzyme incubated with patulin (data not shown).

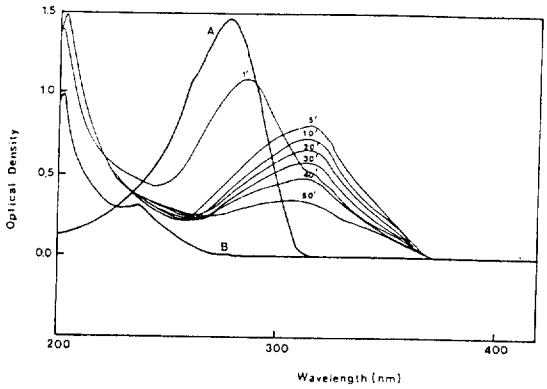


Fig. 4. *Absorption spectra of free cystein (B) and patulin (A).*
The spectra changes of free cysteine reacted with patulin were recorded as a function of time.

Reaction of penicillic acid on GABA transaminase

At similar concentrations of penicillic acid as patulin did not show any inhibitory effect on GABA transaminase, although slight inhibition (15%) was obtained at much higher concentration of 5 mM (Table 1). It has been reported that the terminal unconjugated double bond of penicillic acid could also be a site of interaction with thiols and further shown that the penicillic acid might interact with amino groups (2). According to our results, however, we might suggest that the -SH group on the enzyme was not reacted with penicillic acid because we have already shown that there are two cysteinyl residues critically connected with catalytic activity of the GABA transaminase (6, 16).

We investigated the reaction of penicillic acid with lysyl residues of the enzyme by using the apo- and reduced enzymes. Apo- and reduced forms of the enzyme don't have any catalytic activities because apoenzyme loses the cofactor and the cofactor of reduced enzyme is modified. Therefore, two lysyl residues connected with catalytic activity are exposed on apoenzyme and one lysine residue of second catalytic site of reduced enzyme is exposed, respectively.

Preincubation of penicillic acid (5 mM) with the reduced enzyme shows competitive inhibition against natural cofactor, PLP (Table 2). Similar results were observed when the reconstituted enzyme (reduced enzyme+PLP) was inhibited by penicillic acid. These results indicated that the penicillic acid competed with natural cofactor PLP for binding to lysyl residue on the catalytic domain of the enzyme. In order to confirm those results, we measured the inhibitory effect of reconstitution of apoenzyme. Apoenzyme preincubated with PLP was also inhibited by penicillic acid (Table 2). Those lines of evidences suggest that lysyl residues connected with catalytic activity might be modified by penicillic acid.

Table 1. *Inactivation of GABA transaminase by patulin.*

Reaction mixture	Specific activity (units/mg)	% Specific activity
Enzyme	20.0	100
Enzyme + patulin	2.0	10
Enzyme + α-ketoglutarate + patulin	19.4	97
Enzyme + penicillic acid	17.0	85

Concentration of enzyme, α-ketoglutarate, patulin, penicillic acid in the reaction mixture are 10^{-5} M, 5×10^{-3} M, 5×10^{-4} M, 5×10^{-3} M, respectively. Each reaction was carried out for one hour at pH 7.0 and 25°C.

Table 2. *Effect of penicillic acid (PA) (5 mM) on the reconstitution of reduced and resolved GABA transaminases (10 μM each) with 200 μM of PLP (pyridoxal-5-P).*

Samples	Reconstituded catalytic activity (%)
Native GABA-T	100
Reduced GABA-T	0
Reduced GABA-T+PLP	90
Reduced GABA-T+PA+PLP	35
Reduced GABA-T+PLP+PA	50
Apo GABA-T	0
Apo GABA-T+PLP	100
Apo GABA-T+PA+PLP	40
Apo GABA-T+PLP+PA	51

DISCUSSION

Since abnormal levels of neurotransmitter GABA in brain have been associated with a variety of neurological disorders including Huntington's disease, epilepsy and Parkinsonism, it is important to know the structural and the catalytic behavior of the GABA transaminase involved in the degradation of GABA.

The GABA transaminase isolated from pig brain contains sulfhydryl and lysyl residues which are closely related to the catalytic function of the enzyme (6, 15). In the current study, the enzyme was inactivated by the mycotoxin patulin, and this inhibitory effect was prevented completely by the substrate, α-ketoglutarate, but no protection was afforded by the amino substrate GABA (4-aminobutyrate). This inactivation was easily reversed by the reducing agent, dithiothreitol. These results indicated that critical sulfhydryl residues connected with catalytic activity must be

located close to the substrate binding site of the enzyme. The fact that patulin is readily inactivated by thiols (11) has led to the hypothesis that the mode of action of this mycotoxin is due to their interaction with the -SH residues in the enzyme. The results of our studies raise some questions about the role of the reactive sulfhydryl groups in enzyme function. Two distinct possibilities emerge. The sulfhydryl groups are essential for catalysis, so they participate in some catalytic events. Alternatively, blocking of the reactive sulfhydryl groups triggers a conformational change which affect the catalytic site domain. The first interpretation seems to be consistent with the protective effect exerted by α-ketoglutarate. Binding with α-ketoglutarate can also result in stabilization of protein conformation which are no longer accessible to attacking sulfhydryl reagents. Another interesting point is that even though the patulin is most effective in inhibiting the growth of tissue culture or microorganisms and highly effective in suppressing the formation of urine in mice, little is known about the neurological effect of this mycotoxin. Therefore, it is of interest that the enzyme metabolizing the inhibitory neurotransmitter GABA in the central nervous system can be completely inhibited by the mycotoxin patulin.

Penicillic acid did not show any inhibitory effect at the physiological concentration, but a small inhibitory effect at higher concentration of 5 mM can be demonstrated. It has been already reported that patulin has higher affinity for the dehydrogenase (alcohol and lactate dehydrogenase) than penicillic acid. Furthermore, less patulin than penicillic acid was required to cause enzyme inhibition. Those reports parallel the *in vivo* pattern of biological effects in mice and rats, in which the carcinogenic or lethal dose of patulin is much lower than that of penicillic acid (13).

We have shown that there are powerful evidences that lysyl residue of the catalytic domain reacts with penicillic acid. Penicillic acid competes with natural cofactor PLP for binding to lysyl residues in the catalytic domain of the GABA transaminase. Our results are consistent with the suggested possibility of an addition reaction between an amino group and penicillic acid (11). There is need for further studies of the exact reaction site including, for example, the isolation of lysine- or cystein-mycotoxin adduction from the inactivated enzyme and the sequence of mycotoxin containing peptides. Furthermore the neurological effects by these mycotoxins should be studied under *in vivo* conditions. For instance, after intramolecular injection of these mycotoxins to mouse, we may be able to measure the change of GABA level in brain tissue. Moreover, whether the convulsion or seizure including any other abnormal neurological symptom might be observed with these mycotoxins.

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(Received April 9, 1993)
(Accepted April 20, 1993)

초 록: 뇌의 γ-Aminobutyrate Transaminase에 대한 Mycotoxin의 저해작용

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포유동물들의 뇌조직에서 주 억제성 신경전달물질인 GABA의 분해를 촉매시키는 효소인 GABA transaminase가 mycotoxin인 patulin에 의하여 효소 활성도가 저해되었다. 이 저해작용은 효소의 기질인 α-ketoglutarate에 의하여 protection되었으며, dithiothreitol(DTT)에 의하여 저해되었던 효소의 활성도가 복구된 것으로 보아 patulin이 효소의 활성 부위에 있는 sulfhydryl 잔기와 반응한다는 것을 알 수 있었다. 한편 또 다른 mycotoxin인 penicillic acid는 효소의 보조인자인 pyridoxal-5-phosphate(PLP)가 환원되어 있는 형태의 효소(reduced enzyme)나 보조인자를 제거시킨 형태의 효소(apoenzyme)들의 reconstitution을 저해하는 것으로 보아 이 mycotoxin은 효소의 활성 부위의 lysyl 잔기와 반응한다는 것을 추측할 수 있었다. 그러므로 효소의 활성부위에 위치한 중요한 sulfhydryl 그리고 lysyl 잔기가 mycotoxin인 patulin과 penicillic acid와 각각 반응하여 효소의 활성도를 저해시킨다고 볼 수 있다.