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Original Contribution

FREE RADICAL FORMATION MIGHT CONTRIBUTE TO THE SEVERE AMATOXIN HEPATOTOXICITY

A. Zheleva [1*], V. Gadjeva [1], M. Zhelev [2]

[1]Department of Chemistry and Biochemistry, Medical Faculty, Thrakia University, 11 Armeiska Str. 6000 Stara Zagora, Bulgaria,
[2]Corpora Med EOOD, 6000 Stara Zagora, Bulgaria

ABSTRACT

Our former studies demonstrated that the lipid peroxidation process took place in the liver of mice treated by mushroom hepatotoxin alpha amanitin and in advance application of silybinin decreased the levels of lipid peroxidation products down to those of the control mice. It is known, that damages on antioxidant enzyme defense including enzymes as superoxide dismutase, catalase, gluthation peroxidase might lead to generation of reactive oxygen species followed by increase of lipid peroxidation products levels. Bearing in mind that catalase is an enzyme with high activity in hepatocytes and plays an important role in scavenging of H2O2 it was of interest to study in vitro the effect of mushroom hepatotoxins, alpha and beta amanitin on its activity. Catalase activity was determind by a spectrophotometrical method, following the decrease in the initial H2O2 concentration at 240 nm for 60 s after addition of incubated mixtures of the corresponding amatoxin plus enzyme. After 3 h of incubation at 370C a considerable decrease in enzyme activity was found for both mixtures when compared to that of the single incubated enzyme. It was also found that beta amanitin inhibited catalase activity in a concentration dependent manner. Based on our present results and formerly reported studies we have made an assumption to explain more precisely the severe amatoxin hepatotoxicity.

Key words*:* Alpha amanitin, Beta amanitin, Catalase, Antioxidants, *Amanita phalloides* mushroom

INTRODUCTION

Powerful mushroom hepatotoxins; alpha and beta-amanitin are bridged cyclopeptides, slightly differing in their structures (Figure 1). They were found in a few *Amanita* mushroom species, mainly *Amanita phalloides* (1, 2). It was demonstrated that the basic molecular mechanism of their toxicity was due to the inhibition of RNA polymerase II of the eykariotic cells (3, 4). They cause dramatic toxic consequences mainly within the liver (5) and kidney (6). However, for the different amatoxins (alpha-, beta-, etc amanitin) there is no direct correlation between *in vivo* LD_{50} and inhibitory constants (Ki) *in vitro* (5) determined. Several mechanisms have been already proposed to explain the difference between these parameters (7, 8).

Our formerly reported studies demonstrated that LPO process took place in the livers of alpha-amanitin poisoned mice (8). It is known that *in vivo* increased levels of LPO products might be due to increased levels of reactive oxygen species (ROS) as a result of damages on antioxidant enzyme defence including enzymes as superoxide dismutase, gluthatione peroxidase, catalase. Catalase (CAT) is an enzyme found principally in peroxisomes and to a lesser extent in the cytosol and microsomal fractions of the cell and its highest activities are in the livers, kidneys and red blood cells of mammals (9). Because of the important role of this enzyme in the process of scavenging of H_2O_2 the investigation of possible changes in CAT activity under influence of amatoxins (alpha- and beta-amanitin) seems to be essential.

MATERIALS AND METHODS

Alpha and beta amanitin were isolated from *Amanita phalloides* according to a procedure described previously (10, 11) and compared to the standard toxins purchased from Sigma Chemicals Co (St Louis, USA). Bovine CAT was purchased from Sigma Chemical Co St. Louis, USA. H_2O_2 was purchased from Fluka

*Correspondence to: Associate Professor Dr Antoaneta Zheleva, PhD, Medical Faculty, 11 Armeiska Str., Trakia University, 6000 Stara Zagora, Bulgaria, Tel: + 359 42 28 19 227; fax: +359 42 600 705 E-mail: zheleva@mf.uni-sz.bg

Chemie, Switzerland. TLC assay was performed on Silica Gel F254 plates from Sigma Chemicals Co St. Louis, USA. All other reagents were of the best quality commercially available. UV spectrophotometric studies were performed on a Pharmacia LKB Ultrospec spectrophotometer (Sweden).

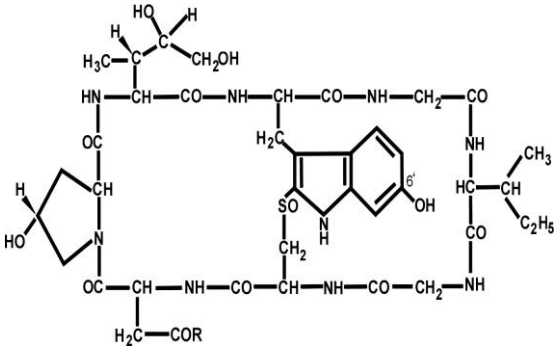

Figure 1. Chemical structures of alpha amanitin ($R = NH_2$) and beta amanitin (R = OH)

Enzyme assay

CAT activity was determined following the decrease in the initial H_2O_2 concentration (30 mM) at 240 nm at 25°C in 60 s according to Johansson and Borg (12) with some modifications. That is, incubated at 37°C for 3 h mixtures of 100 μl CAT (5 mg/ml) plus corresponding amatoxin (0.016 μM or 0.032 μM) were placed in a cuvette in PBS buffer (50 mM) to a final volume 2 ml. 1 ml of H_2O_2 was added and a decrease of its absorbency at 240 nm occured in 60 s. Activity of the standard of CAT after single incubation under the same experimental conditions has also been studied. A sample containing corresponding amatoxin plus H_2O_2 was used as a blank.

TLC assay

The samples of alpha and beta amanitin and mixtures of the corresponding amatoxin plus CAT incubated at 37°C for 3 h were spotted on TLC plates and eluted with a mobile phase containing chloroform/methanol/acetic acid/water (75:33:5:7.5, v/v/v/v). TLC plates were stained with trans cinnamaldehyde (1 % in methanol) and immediately exposured to hydrochloric acid vapours (13).

RESULTS

Results from CAT activity of the incubated mixture of the corresponding amatoxin plus enzyme compared to that of the single incubated standard of CAT, which was considered as 100 % activity are presented on Figure 2. After incubation of the toxins plus CAT a considerable decrease in the enzyme activity was registered in both mixtures. At a concentration of 0.016 μM of the amatoxins, the persentage of CAT activity for the beta amanitin mixture was significantly higher than that of the alpha amanitin mixture (mean 84.17±3.83 %. vs 68.61 ±10.03 %, p=0.01, t test). At a concentration of 0.032 μM, the persentages of inhibition were similar for both toxins (mean 69.53±4.20% for alpha amanitin vs 62.12 ±5.09% for beta amanitin, $p<0.05$, t test). It is also evident that beta amanitin inhibited CAT activity in a concentration dependent manner (mean 84.17% for concentration of 0.016 μM vs 62.12% for concentration of 0.032 μM, p=0.001), while the change in alpha amanitin concentration did not affect that activity (mean 68.61 % for the concentration of 0.016 μM, vs 69.53% for the concentration of 0.032 μM, $p>0.05$, t test). On TLC plate the typical purple colour spots (closely related to the presence of 6'-hydroxyindole chromophore moiety) with equal Rf values were visualized (5) for pure amatoxins and for their mixtures with CAT, too (results are not shown).

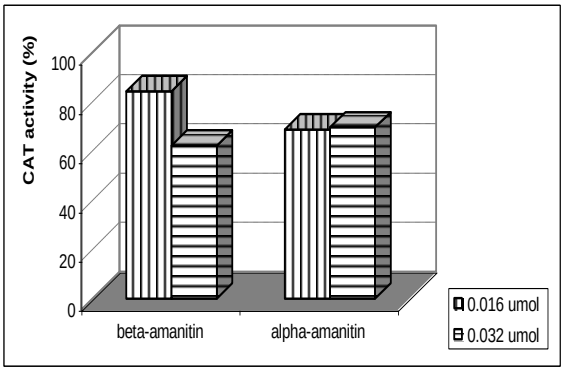

Figure 2. Effect of alpha and beta amanitin on CAT activity after 3 h incubation at 37°C. CAT activity of the incubated mixture of corresponding amatoxin plus CAT was compared to that of the incubated single standard of CAT, which was considered as 100 % activity.

DISCUSSION

Bukovska et al., (14) have found a decrease in CAT activity under the influence of both chlorophenols 2,4-DCP and 2,4,5,-TCP. They have explained that effect by the blockage of the sulphydryl residues and thus the spatial structure of the enzyme. Sulphuric amino acids play very important role in the activity of CAT (15). To explain the inhibitory effect of the amatoxins at our experimental conditions we accept a similar blockage of sulphydryl residues of CAT because of the presence of similar structure moieties in both

amatoxins (see Figure 1, phenolic group in 6'-hydroxyindole moiety). It should be mentioned that the latter structure was still preserved during incubation of the amatoxins plus CAT, confirmed by TLC assay. Another explanation concerning the amatoxin inhibitory effect might be related to blocking the access of H_2O_2 to CAT heme Fe pocket (16). The catalytic process of CAT is thought to occur in two stages:

H_2O_2 +Fe(III)-E $\rightarrow$ H_2O + O=Fe(IV)-E (1)

H_2O_2 + O=Fe(IV)-E $\rightarrow$ H_2O + Fe(III)-E (2)

To be realized the first stage of this process H_2O_2 should enter the heme cavity. Upon entering the latter, H_2O_2 must interact with His^{74} and Asn^{147} (16). We assumed that closely to the funnel–shaped channel descending toward the heme, hydrogen bonds between the CAT and amatoxins might be formed during the incubation. A formed complex between the enzyme and corresponding amatoxin probably hampers the access of H_2O_2 to the above mentioned aminoacid moieties. The difference in the degree of the inhibitory effect of both amatoxins demonstrated by this study might be due to a slight difference in their chemical structures (5). The concentration dependence for the beta amanitin inhibitory effect and the lack of a similar dependence for alpha amanitin demonstrated by the present study could be explained to a some extent by the better hydrophility (see Figure 1) of beta amanitin in comparison to that of the other amatoxin (5). This finding is also supported by the fact that CAT heme channel is lined with hydrophilic residues at its entrance (16). Our formerly *in vitro* experiments showed that alpha and beta amanitin could interact with the stable free radical 1,1-diphenil-2-picrylhydrasyl (17). We also established that alpha amanitin decreased DPPH absorbency at 517 nm in a concentration dependent manner (8). When a free radical reacts with a nonradical compound other free radicals might be formed. This enables free radicals to induce chain reactions - for example, LPO involving polyunsaturated fatty acids (18). EI-Bahay et al. (19) have shown that in rat hepatocyte cultures LPO is low when alpha amanitin was alone even at 36 h but markedly increased by cotreatment with tumor necrosis factor-alpha (TNF-α). They have also found that antioxidant silybinin prevents the effect of TNF-α indicating an involvement of ROS. Our previous *in vivo* experiments demonstrated that within 20 h alpha amanitin could initiate LPO in the livers of mice. Since, silybinin is able to protect the cell membrane from radical – induced damage and also to blockade the uptake of toxins, like amatoxins (20, 21) we administrated this antioxidant into the mice before alpha amanitin treatment and found a decrease in LPO products levels down to the controls of the non poisoned mice (8). By ESR spectroscopy method Ghibaudi et al., (22) have studied the ability of the lactoperoxidase/H_2O_2 system to generate free radicals from the estrogens, diethylstilbestrol, 2-hydroxyestradiol and 4-hydroxyestradiol. To investigate the free radical origin the same authors have used UV-visible spectroscopy and established that the cores of those estrogens sensitive to oxidation were the phenolic groups in their structures. Formerly, by UV-visible spectroscopy we have shown that alpha amanitin was sensitive to oxidation by a system of lactoperoxidase/H_2O_2 and assumed formation of free radical intermediates and in particular sulphinyl radicals from the amatoxin (23).

Based on the present *in vitro* experiments and previously reported studies we have suggested the following assumption to explain more precisely severe amatoxin hepatotoxicity: As alpha and beta amanitin could participate in free radical reactions, penetration through liver cell membranes and entering the hepatocytes is possible by themselves to transform into phenoxyl and/or sulphinyl free radical intermediates. The latter might stimulate the creation of ROS ($^{\cdot}O_2^-$ H_2O_2, $^{\cdot}OH$) and as a result chain reactions to be induced in the membrane polyunsaturated fatty acids. Moreover, if amatoxins could *in vivo* inhibit some of the antioxidant enzymes like CAT, this might additionally contribute to an increase in the levels of ROS and LPO products. In conclusion, we also consider that other antioxidants (not only silybinin) might be selected for an earlier and more adequate proper therapy of patients poisoned by *Amanita phalloides* mushroom.

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