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Original Contribution

ANTIOXIDANT PROPERTIES OF AMANITA PHALLOIDES MUSHROOM TOXINS

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SUMMARY

Alpha amanitin and phalloidin are powerful hepatotoxins that belong to the main classes of toxins namely, amatoxins and phallotoxins, isolated from the deadly poisonous mushroom *Amanita phalloides*. It is well known that disruptions of antioxidant enzyme defense that include superoxide dismutase (SOD), catalase, glutathione peroxidase lead to generation of reactive oxygen species, followed by increase in the products of lipid peroxidation. Our previous studies showed that alpha amanitin and phalloidin could inhibit catalase activity. These generated the interest to study the in vitro behaviour of both toxins on SOD activity using spectrophotometry. Our result showed some slight increase of SOD after incubation of the enzyme and toxins. Superoxide anion scavenging activity (SSA) of alpha amanitin and phalloidin was also studied. It was established that both toxins, like the enzyme, exhibit SSA activity. It was obvious that at our experimental conditions alpha amanitin and phalloidin behaved as antioxidants.

Key words: Alpha amanitin, phalloidin, superoxide anion scavenging activity.

INTRODUCTION

Amatoxins and phallotoxins are powerful hepatotoxins. Amatoxins - alpha and beta amanitins, and phallotoxins - phalloidin are bridged oligopeptides (**Figure 1, Figure 2**) found in a few Amanita mushroom species, mainly *Amanita phalloides* (1, 2)

Amatoxins elicit toxicity by inhibiting the RNA polymerase II of the eukaryotic cells (3,4). They cause dramatic toxic consequences mainly within the liver (5) and kidney (6). However, the different types of amatoxins (alpha-, beta-, etc amanitins) do not show any straight correlation between *in vivo* LD_{50} and inhibitory constants (Ki) obtained from *in vitro* studies (5).

Hepatotoxic effects of the phallotoxins including phalloidin, have been attributed to their specific binding to F-actin, thus strongly stabilizing the structure of the assembled filaments (2). Later studies showed their ability to deplete the mitochondrial Ca^{2+} pool after treatment of intact hepatocytes by phalloidin. This finding was related to the primary toxic effects of phalloidin poisoning (7). Moreover, Ca^{2+} release from mitochondria induced by prooxidants has been discussed in details (8, 9).

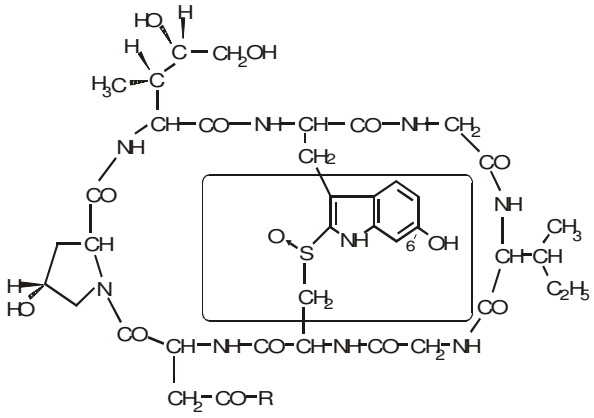

Figure 1. Chemical structure of alpha amanitin (R = NH_2)

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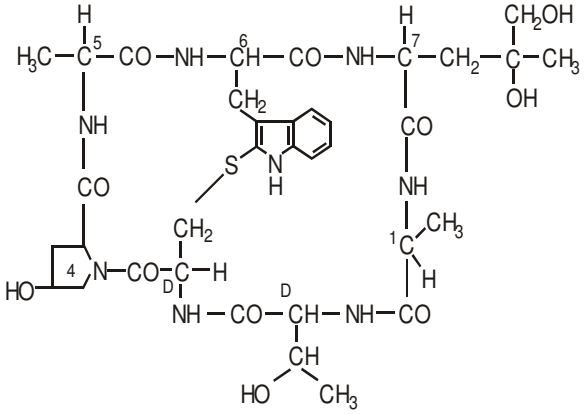


Figure 2. Chemical structure of phalloidin

Our previous studies have demonstrated that LPO process took place in the livers of alpha-amanitin poisoned mice (10). 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 damage to the antioxidant enzyme defense system comprising essentially of superoxide dismutase, gluthatione peroxidase and catalase. Using *in vitro* studies we have demonstrated that alpha amanitin, beta amanitin and phalliodin could inhibit catalase (CAT) activity to a considerable degree (11). Based on the above, it became necessary to study the *in vitro* effect of alpha amanitin and phalloidin on superoxide dismutase (SOD) activity, as well.

MATERIALS AND METHODS

Alpha amanitin, beta amanitin, phalloidin, enzyme SOD and all other reagents were purchased from Sigma Chemical Co (St. Louis, USA).

All visible and UV spectrophotometrical studies were performed on a Pharmacia LKB Ultrospec spectrophotometer (Sweden).

Enzyme assay

Determination of SOD activity was based on the generation of superoxide anion radical $O_2^{\cdot-}$ in a system containing hypoxanthine/xanthine oxidase in which $O_2^{\cdot-}$ reduced nitroblue tetrazolium to formazan; the absorbance of the latter was measured at 560 nm. SOD activity was determined according to Sun et al., (12) with slight modifications. Briefly, in glass tubes were added consecutively the following reagents: 200 µl hypoxanthine (0.075mg/ml of PBS), 20 µl EDTA (0.085 mM), 400 µl NBT (0.05mg/ml of PBS), and then incubated for 3 h at 37°C. Mixtures of SOD (100 µl, 0.0011 mg/ml) plus alpha amanitin [31.2 µl (0.016 µM), 62.4 µl (0.032 µM)], or phalloidin [100 µl (0.057 µM)], 850 µl, 820 µl or 780 µl PBS buffer and 400 µl xanthine oxidase (0.25 mg/ml PBS) were added to a final volume of 2 ml. Activity of control standard SOD after incubation at the same experimental conditions was also studied. Tubes were stirred, stoppered and incubated at 37°C. Absorbance of the standard incubated enzyme was taken as 100 % activity. After 20 min incubation, samples were put on ice and absorbance at 560 nm was measured. Control sample did not, of course, contain either toxin or enzyme. Their quantities were replaced by equivalent amounts of PBS buffer. Absorbance at 560 nm of the controls presented the quantity of the formed superoxide anion ($O_2^{\cdot-}$) taken as 100 %.

RESULTS AND DISCUSSION

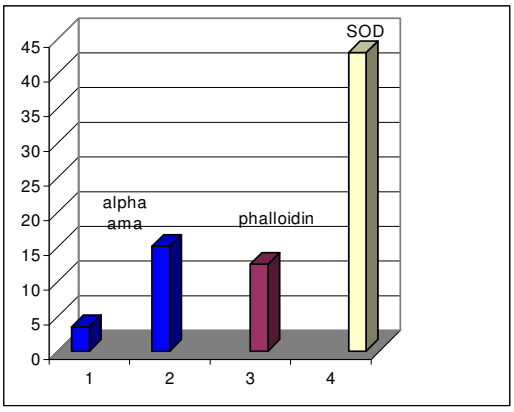
Results from SOD activity of the incubated mixtures of corresponding amatoxin concentration or phalloidin plus enzyme compared to that of the SOD standard showed some increase in SOD activity. At a concentration of 0.016 µM of the alpha amanitin, strength of SOD activity of the mixture was 107.99 ± 5.83 %. At a concentration of 0.032 µM, strength of SOD activity of the mixture was 103.44 ± 13.59 %. At a concentration of 0.057 µM of the phalloidin, strength of SOD activity of the mixture was 105.26 ± 6.53 %. These results demonstrated slight activating effect of the toxins towards the enzyme. To check whether that effect was due to superoxide anion scavenging activity (SSA) of the toxins we studied absorbance of samples containing nonincubated toxin (0.016 µM and 0.032 µM for alpha amanitin and 0.057 µM for phalloidin) alone in comparison with that of the control samples and that of nonincubated SOD. Results obtained (see **Figure 3**) showed that at a concentration of 0.016 µM alpha amanitin exhibited 3.50 ± 1.47 % SSA activity, while at a concentration of 0.032 µM this activity was 15.07 ± 3.97 %. Phalloidin exhibited 12.49 ± 2.36 % SSA activity; at the same time nonincubated SOD exhibited 42.95 ± 3.94 % SSA activity. As is seen SSA activity of the amatoxin depended on its concentration. Based on the results obtained, activating abilities of alpha amanitin and phalloidin towards SOD could be explained by their SSA activity at our experimental conditions. Lack of the additive activating effect might be explained by possible formation of the complex between

the toxins and enzyme during the incubation which hampered active toxin groups (probably phenolic group in 6'-hydroxyindole moiety and/or sulphoxide from the bridge for alpha amanitin and sulphur from the bridge structure for phalloidin, see **Figure 1**, **Figure 2**) to exhibit their SSA activity completely.

Figure 3. SSA activity at concentrations of alpha amanitin (**1** - 0.016 μM; **2** - 0.032 μM), phalloidin (**3** - 0.057 μM) and SOD (**4** - 0.0011 mg / ml)

CONCLUSION

Our recent *in vitro* studies had demonstrated



that both toxins inhibit CAT activity. Our present studies have shown that alpha amanitin and phalloidin possessed SSA activity. It could be suggested that alpha amanitin and phalloidin exhibited prooxidant or antioxidant properties at our experimental conditions. That means they could be involved in free radical reactions. It seems very likely that they penetrate liver cell membranes and enter the hepatocytes where they constitute reactive free radicals. This might stimulate creation of ROS ($^{\cdot}O_2^-$ H_2O_2, $^{\cdot}OH$) and as a result induce chain reactions in the membrane polyunsaturated fatty acids. Moreover, if both toxins could inhibit *in vivo* some of the antioxidant enzymes, such as CAT, they could additionally lead to increase in the levels of ROS and LPO products and contribute to their severe hepatotoxicity.

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