CHARACTERIZATION AND PARTIAL PURIFICATION OF THE TOXIN OF LEPIOTA MORGANII

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FREDERICK I. EILERS and LOUIS R. NELSON. Characterization and partial purification of the toxin of Lepiota morganii. Toxicon 12, 557-563, 1974.—The toxin of L. morganii is present throughout the mushroom, but most concentrated in the cap tissue. It is heat labile, losing all detectable activity in 30 min at 70°. The toxin is proteinaceous, with an approximate molecular weight in excess of 400,000 Daltons and composed of sub-units of approximately 40,000-60,000 Daltons. Attempts to degrade the toxin with proteolytic enzymes were partially successful in that some loss of activity was effected. The toxin administered to mice and chicks produced the following symptoms: hesitancy to move, drowsiness, tremors, abdominal contractions (forced breathing), and death.

INTRODUCTION

THE POISONOUS nature of the green spored Lepiota, L. morganii (Peck) Sacc. has been known for almost 100 years. Human ingestion causes gastro-intestinal poisoning accompanied by vomiting, diarrhea, occasional nausea, unusual thirst, and extreme sensitivity to light and sound (GRAFF, 1927; MCCARTER, 1959). Poisoning is rarely fatal and usually is self-limited with recovery occurring without treatment in 1 or 2 days.

This mushroom is most common in warmer climates, but can be found in much of the United States (BESSEY, 1939; SMITH, 1936). It has also been reported in British Guiana, Brazil, the West Indies, the Philippine Islands, Tahiti, and Central Africa (MCCARTER, 1959; SMITH, 1936; GRAFF, 1927). In Florida, the mushroom occurs most often in well-maintained lawns. As an example of its abundance, we have collected 6-8 kg of fresh weight of mushrooms per week throughout the summer of 1972 from the main area of the University of South Florida campus (approximately 72 hectares). Due to this abundance, frequent accidental ingestions occur.

While information is available on its geographical distribution and clinical symptoms, little is known about the nature of the toxin. The effects of the toxin in mice has been described by FLÖCH et al. (1966). In this report we describe some of the properties of the toxin and attempt to quantify the effect of the toxin on mice and chicks.

MATERIALS AND METHODS

Lepiota morganii (Peck) Sacc. (used throughout this study) were collected between June and November, 1972 and 1973, on the campus of the University of South Florida. The mushrooms were either used immediately or stored at 6° for no more than 14 days before use. Toxin extracts were prepared by homogenizing mushroom caps in distilled water using a Waring blender. This initial extract was sometimes used directly, but in most
instances further purified by: centrifugation at 15,000 g for 30 min to remove cell debris, precipitation by ammonium sulfate [35 g (NH₄)₂SO₄ per 100 ml extract], a second centrifugation at 15,000 g for 30 min, then dialysis of the precipitate overnight in distilled water and finally centrifugation at 15,000 g for 30 min. The resulting supernatant contained the toxin.

Initial extractions of mushrooms with other solvents were performed by homogenizing caps in each of the following solvents: 95 per cent ethanol, diethylether, acetone, chloroform, and methylene chloride. The extracts were then centrifuged and the supernatant retained. In the cases of three of the solvents—diethylether, chloroform and methylene chloride, the organic layer was separated from the aqueous layer. The organic solvents were then removed by flash evaporation at 40° and the residue immediately resuspended in a volume of water equal to initial volume of the organic solvent.

The toxin was assayed by peritoneal and subcutaneous injections and oral administration at various concentrations into mature female white mice (Carworth CFW, average weight 38.5 g). The maximum aliquot used was 1.0 ml, and each sample tested was injected into a group of 4 or 5 mice or chicks. Injections were made with 1 ml tuberculinsyringes and 25 or 26 gauge needles, while forced oral administrations were made with oral feeding needles. Death of the animal in one or two days after treatment was used as positive indication of the presence of the toxin.

In experiments attempting to degrade the toxin, the following proteolytic enzymes were used: trypsin (Type I), pepsin (1:60,000), protease (Type V), all from Sigma; pronase (Grade B) from Calbiochem; and rennin (Grade I) from Miles-Seravac. To evaluate the effect of the enzymes on the toxin, unbuffered extracts were adjusted to the optimal pH for each enzyme by the addition of 6 N HCl or NaOH. To this was added 1 mg of enzyme per ml of extract (units of activity varied depending on the enzyme) and the mixture was allowed to incubate at 22° for up to 24 hr. The pH was then readjusted back to the initial value (5.6-5.9) and aliquots from 0.1 to 1.0 ml were injected into mice. Control mice were injected with pH adjusted extracts or solutions of enzyme.

The Bio-Gel P series gels, used in molecular weight estimations, were equilibrated with 0.05 M phosphate buffer, pH 6.8. The column size was 15 x 2 cm, and the void and exclusion volumes of each column were determined with blue dextran (molecular weight 2 x 10⁶).

Dogs were anesthetized with pentobarbital sodium (30 mg/kg) and tracheotomized. Carotid blood pressure was measured using a Grass kymograph and a Strathamp pressure transducer. The femoral vein was cannulated with polyethylene tubing for the injection of the various agents.

RESULTS

The susceptibility of mice and chicks to L. morganii toxin

To determine the susceptibility of mice and chicks to the toxin, groups of 4 mice and 5 chicks were treated with various concentrations of toxin in one of three ways: (1) orally by force feeding (mice and chicks), (2) injection into the peritoneal cavity (mice and chicks), (3) subcutaneous injection (mice only). In both animals, force feeding did not kill or cause noticeable distress. Subcutaneous injections in the flank or back of the neck of mice had no significant ability to kill except that one mouse died at the highest concentration. The mice developed skin abscesses in 10-14 days. In mice, i.p. injections caused them to sit in a crouched position, to be hesitant to move, and caused tremors and
abdominal contractions (forced breathing). The onset of these reactions occurred consistently in 5-15 min depending on dosage and continued up to 2 days, often resulting in death. However, animals injected with lower concentrations recovered spontaneously. These reactions to the toxin appeared somewhat similar to those described previously by Floch et al. (1966).

Mice injected with edible mushroom extracts (Agaricus bisporus) at 5 times the concentration used in most Lepiota extracts, were used as controls. These mice exhibited no toxic symptoms. Additional controls consisted of mice injected with Lepiota extracts which had been detoxified by heat or low pH (2.0), or the supernatant from the ammonium sulfate precipitation. These also caused no toxic symptoms.

The approximate minimum fresh weight of mushrooms required to extract sufficient toxin to be lethal to about 50 per cent of the mice, is 0.04 g for crude uncentrifuged extracts, 0.33 g for centrifuged extracts, and between 0.6 and 1.6 g for extracts precipitated by ammonium sulfate, dialyzed, and recentrifuged. This indicates that considerable toxin loss occurred in the preparative steps.

The reaction of chicks to Lepiota toxin was somewhat similar to mice in that movement ceased and hyperventilative contractions occurred. The chick's abdominal wall was damaged by the toxic material injected and usually ruptured. The rupture of the wall allowed the viscera to herniate before the birds died. The chicks also seemed to go into a stupor, with the head nodding as though they were falling asleep. The sensitivity of chicks to the toxin was similar to that of mice in that with an ammonium sulfate precipitated, dialyzed, and recentrifuged extract, approximately half of the animals were killed by an extract equivalent to 0.7 g fresh weight of mushroom. However, the chicks died much faster than mice, many in 15-60 min. As with mice, chicks injected with distilled water or detoxified Lepiota extract exhibited no abnormal reactions.

Localization of toxin

To determine the gross distribution of the toxin in the plant, mature mushrooms were divided into stipes, cap tissue minus gills, gill tissue (containing spores) and basidiospores. Essentially identical extracts of each tissue were injected into mice and the results are shown in Table 1. The toxin was found in all parts of the mushroom; however, based on lethality of fresh weight extracts, the stipes and gills appeared to contain less toxin. Cap tissue contained sufficient toxin to kill at half the lethal gill or stipe concentration. The toxin level in spores was more difficult to compare to that in other mushroom parts as the fresh weight was essentially the dry weight. Transposing from the fresh to dry weight at 55°C, 1 ml of stipe, gill or cap extract contained the toxin derived from 58, 53 and 44 mg dry tissue, respectively. Consequently, cap tissue extracts were lethal at an equivalent of 17.6 mg dry weight compared to spores at 50 mg, stipe extracts at 58 mg equivalent, and gills at 40 mg equivalent. Based on this data, stipe tissue was no longer used in the preparation of extracts. However, gill tissue was utilized because of the difficulty in separating it from the rest of the cap.

In immature white spored mushrooms, the distribution of toxin was similar to mature green spored ones. Stipe tissue contained the least toxin, followed by gill tissue, with the highest concentration in cap tissue.

Solubility and extraction of toxin

To aid in characterization and purification of the toxin, attempts were made to extract
Groups of four mice were given i.p. injections of extracts of the various mushroom parts. Recorded below are the number of mice killed by each injection within 48 hr.

| Volume of extract (ml) | Approximate fresh wt. equiv. (g) | Stipe | Cap | Gills | Spores
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<tr>
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<td>0</td>
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<tr>
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<td>0.26</td>
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<td>0</td>
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<tr>
<td>0.5</td>
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<td>1</td>
<td>4</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>0.75</td>
<td>0.49</td>
<td>0</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>1.0</td>
<td>0.66</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>50</td>
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</table>

The results shown in Table 2 indicate no loss of toxin activity at 50° for up to 90 min and little loss at 60° for up to 30 min after which the 0.6 ml aliquot was no longer lethal. However, the 1 ml dose retained sufficient activity to be 100 per cent lethal for at least 90 min at 60°. At 70°, the toxin was rapidly destroyed losing lethality at 0.6 ml in 5 min and all detectable activity with 1.0 ml in 30 min.

**Heat stability of the toxin**

Previous investigators (Flocit et al., 1966) have indicated that the toxic properties of *L. morganii* were eliminated by boiling, but did not provide more precise data on heat stability. To characterize the toxin, further extracts were heated at 50°, 60° and 70° for various times from 5 to 90 min. After heating, mice were injected with two concentrations: one (0.6 ml) representing the amount just above the minimum dose showing 100 per cent lethality (0.5 ml) for this extract, and a second amount representing the maximum volume which we used in mice (1.0 ml).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>22° (Control)</th>
<th>50°</th>
<th>60°</th>
<th>70°</th>
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<tr>
<td>5</td>
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<td>1</td>
<td>3</td>
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<td>0</td>
</tr>
<tr>
<td>90</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 2. Heat stability of L. morganii toxin*

Groups of four mice were injected intraperitoneally with extracts of toxin heated at 50°, 60° and 70° for up to 90 min. At each time, the mice were injected with a dose of 0.1 ml above that previously determined to be 100 per cent lethal (0.6 ml) and the maximum dosage used (1.0 ml). Listed below are the number of mice killed of the group of 4 injected.
Effects of proteolytic enzymes on the toxin

Of the enzymes used, only pepsin caused an apparent loss of toxicity as indicated by the greater number of mice which survived after injection with pepsin treated abstracts compared to those injected with control extracts (Table 3).

In comparing the effect of pepsin on the toxin, we found that control extracts which received a pH adjustment to 2-0, lost considerable activity. In the experiment reported in Table 3, the extract initially was 100 per cent lethal at 0-2 ml. However, after incubation at pH 2-0 for 1 hr the 100 per cent lethal dose was 0-75 ml. In other experiments in which the pH 2-0 incubation varied from 2 to 8 hr, an increase of 3-10 times the initial dose was required to kill the mice. Because of this acid lability of the toxin, there is less of a differential in toxicity between pepsin treated and control extracts than would be expected.

<table>
<thead>
<tr>
<th>Volume of toxin extract injected (ml)</th>
<th>Pepsin treated extract</th>
<th>Control extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice injected</td>
<td>Mice killed</td>
</tr>
<tr>
<td>0-2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>0-3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0-4</td>
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<td>2</td>
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<td>0-75</td>
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<td>4</td>
</tr>
<tr>
<td>1-0</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Partial purification of the toxin

Solubility in water and heat lability suggested that the toxin was proteinaceous and hence could be purified by standard protein procedures. We have partially purified our extracts using the procedure outlined earlier, which eliminated only cellular debris and low molecular weight compounds.

Extracts prepared in water contained considerable quantities of phenolic compounds and turned dark brown in several min. This discoloration was largely prevented by the addition of 10⁻³ M phenylthiourea and 10⁻⁴ M sodium dithionite. After dialysis these extracts contained about one-third of the polyphenolic discoloration.

When injected intraperitoneally into mice, there was no detectable difference in toxicity between extracts made with or without the addition of dithionite and phenylthiourea. For example, groups of 4 mice were each injected intraperitoneally with aliquots of extracts made with and without the chemicals. In both cases, mice receiving a dose equivalent to 0-28 of fresh weight of mushroom (0-1 ml) lived, while all those receiving dosages of 0-84 g (0-3 ml) or greater, died.

However, when 5-0 ml of toxin extract (9-6 g/ml fresh weight equivalent, 0-4 ml lethal mouse dose), were injected intravenously into a dog (female, 11-4 kg), a decrease in mean blood pressure from 168 to 65 and a change in breathing to predominantly abdominal breathing were noted. The injection of norepinephrine (2 mg/kg) after the blood pressure
increased slightly, caused a small increase from 90 to 93 compared to the initial response before toxin administration of from 162 to 173. On the other hand, epinephrine (2 mg/kg) injected before the toxin caused a decrease from a mean value of 163–83, and that injected after the toxin caused a decrease from 98 to 60. A second injection of 5.0 ml of mushroom extract resulted in a further decrease in blood pressure from 128 to 37 and the death of the animal. These results indicate that the crude extracts do cause a significant decrease in blood pressure and as suggested by the weak norepinephrine response, have an effect similar to an α adrenergic blockage.

This decrease in blood pressure was further investigated by removing the polyphenolic components by absorption on to DEAE cellulose. The resin was charged with 0.5 M phosphate buffer, pH 6.8 to prevent proteins from being retained. This procedure eliminated almost all of the polyphenols, while the toxicity to mice was unaffected.

When 5.0 ml of this polyphenol free extract (7.6 g/ml fresh weight equivalent, 0.1 ml lethal mouse dose) were injected intravenously into a second dog (male, 11.8 kg) the blood pressure changed little from a mean value of 137–148. The norepinephrine (2.5 mg/kg) response was also unaffected. The change before toxin addition was 138–200 and after addition, it was 140–198. The epinephrine (1.25 mg/kg) response remained as in the first dog, showing a decrease from a mean of 135–107 before toxin injection, and from 145 to 128 after. Injection of 5.0 ml of identical extract, from which the polyphenols had not been removed, caused a blood pressure drop from 132 to 55. These results suggest that the original decrease in blood pressure and the α adrenergic blockade were due to the polyphenols. However, since the polyphenols only appear to have a toxic action when injected intravenously, no attempts were made to remove them completely.

To further characterize the toxin, its molecular weight was estimated using the exclusion limits of Bio-Gel P series gels. Aliquots of polyphenol free toxin extract were placed on columns of Bio-Gel P-30, 60, 100, 150, 200 and 300 having exclusion limits from 40,000 (P-30) to 400,000 Daltons (P-300).

The exclusion volume or that fraction containing material which was too large to be trapped in the gel matrix was determined by adding blue dextran (mol. wt 2 x 10⁶) to each column and measuring the volume of the fraction containing the marker. The toxin was added to each column and the excluded fraction was collected, concentrated to the initial volume by ultrafiltration (Amicon model 10 KA M-50 filter), and injected intraperitoneally into mice. The lethality of these fractions were then compared to that of the original extract. With each column, the toxin was found in the fraction containing the material which was too large to be retained by the gel, indicating an approximate molecular weight of over 400,000 Daltons. Based on lethality, the toxin appeared to be completely eluted from the columns in this single fraction.

The large size of the toxin suggested that it may be composed of a number of small sub-units. This possible polymeric composition was tested by adding 8 M urea in an attempt to dissociate the toxin into sub-units. Control experiments demonstrated that it was possible to incubate the toxin with urea at 6° for 24–72 hr, and remove the urea by dialysis without loss of activity. Aliquots of extract incubated with urea for 24 hr at 6° were applied to P-30, 60, 100 and 150 columns equilibrated with 8 M urea to prevent toxin reassociation while on the column. Again the excluded fraction was collected, concentrated to its initial volume and dialyzed. The fractions from the P-150, 100 and 60 columns did not contain any detectable toxin, while that from the P-30 column did contain toxin. The toxin recovered from the P-30 column was almost equivalent to the starting extract. These data indicate...
that the toxin can be dissociated into small sub-units, and these sub-units are small enough
to be trapped by the P-60 gel but not the P-30 gel. This would set the monomer size at
approximately 40,000 (P-30 gel) to 60,000 Daltons (P-60 gel.)

DISCUSSION

It has been reported that *L. morganii* toxin bore certain similarities in its clinical and
chemical characteristics to those of choline derivatives, aromatic amines, and polypeptides
(FLOCH et al., 1966) as well as muscarin (MCCARTER, 1959). However, based upon results
including the toxin's solubility, heat lability, pepsin and HCl sensitivity, high molecular
weight, and sub-unit structure, it would appear that the toxin could not be a choline deriva-
tive, an aromatic amine or muscarin, but rather a polymeric protein with a molecular
weight in excess of 400,000 Daltons. The monomer size appears to be between 40,000–
60,000 Daltons. It is not known how many monomers comprise the polymer or if they are
identical and active as toxins. Our experiments did not explicitly exclude the possibility
that the toxin was a small molecule attached to a non-functional protein component.
However, heat lability, sensitivity to pepsin and acid, argue against such a combination.
Thus *L. morganii* toxin appears to be considerably different from other mushroom toxins
(BUCK, 1961).

These chemical properties and characteristics suggest reasons why *L. morganii* toxin is
rarely, if ever, fatal. Acid and pepsin lability and perhaps its large size may prevent the
molecule from being absorbed into the blood stream in fatal quantities. In addition,
toxicity is further decreased by cooking.

The reactions of mice in our experiments were seemingly identical to those described
earlier by FLOCH et al. (1966). These symptoms as well as those described in clinical cases
suggest that muscle contractions play a central role. In chicks the situation seems similar,
as muscle contractions may cause the weakened abdominal wall to rupture, allowing the
intestines to descend.

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The animals involved in this study were maintained in accord with the “Guide for Laboratory Animal
Facilities and Care”, as published by the National Academy of Sciences, National Research Council.

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