New Laboratory Scale Purification of β-Amanitin from American *Amanita phalloides*†

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ABSTRACT: A modern laboratory scale method for purifying β-amanitin from the mushroom *Amanita phalloides* is described. The purification involves solubilizing the toxin from dried mushrooms, removing the lipids by chloroform extraction, desalting on Amberlite XAD-4, adsorption chromatography on Sephadex LH-20 at acidic and neutral pH, and ion-exchange chromatography on QAE-Sephadex. The resulting β-amanitin is homogeneous by thin-layer chromatography and is biologically active.

The poisonous olive-green mushroom, *Amanita phalloides*, contains two families of toxic bicyclic peptides, the amatoxins and the phallotoxins (for review, see Wieland & Wieland, 1972). Both have interesting biological and structural properties. The amatoxins contain hydroxylated isoleucine and 6-hydroxytryptophan bridged by a cysteine sulfoxide and are potent inhibitors of eukaryotic RNA polymerases II and III (Cochet-Meilhac & Chambon, 1974). The phallotoxin group contains several novel hydroxylated amino acids and a cysteine bridged to tryptophan by a thioether. Phallotoxins act by binding to F-actin and inhibiting its depolymerization (Lengsfeld et al., 1974).

Virtually all of the purification and chemistry of these toxins has been done by the Wielands and co-workers (Wieland et al., 1949; Faulstich et al., 1975). The purifications have been typically done on an industrial scale starting from mushroom harvests of hundreds of kilograms (for example, Wieland et al., 1958). The usual procedure has been summarized (Wieland, 1967). Several features of this method were necessary because of the large scale; a small laboratory scale purification can be greatly simplified. Moreover, there are some specific problems with the published method. First is the requirement for large volumes of methanol which is both expensive and toxic. Second is the use of adsorption chromatography employing organic solvents such as butanone and acetone. Toxicity and flammability require that such columns be operated in a specially ventilated area. Finally, the yields on silica and alumina were often as low as 70% for a single chromatographic step (Lynen & Wieland, 1937).

With the advent of the Sephadexes, the later stages of the purification were greatly modernized and improved, and detailed procedures for using these adsorbants for purifying some of the minor toxic components have been published (Faulstich et al., 1975; Wieland et al., 1967). However, the use of the Sephadexes for purification of the major toxic components, α- and β-amanitin and phalloidin, has only been alluded to, and details of the complete method have not to date been published.

The need for small quantities of amanitin for biochemical and structural studies made it appropriate to design a modern laboratory scale purification procedure. This article describes a method which minimizes the use of noxious organic solvents, uses only aqueous buffers for column chromatography, and gives excellent yields at each step. β-Amanitin was chosen as the toxin of primary interest in this study for several reasons. It is the predominant amatoxin in American *A. phalloides*, in contrast with the European variety which contains more α-amanitin (Yocum & Simons, 1977). Moreover, β-amanitin can be coupled via a peptide bond to a variety of other molecules and remain biologically active (Faulstich et al., 1974). It is not commercially available.

Experimental Procedures

General

Distilled, deionized water was used in all experiments. All chemicals were reagent grade and all steps were performed at room temperature unless otherwise specified. Sephadex LH-20 (lot no. 104C-0023) and QAE-Sephadex A-25 were purchased from Sigma, analytical silica gel 60 F-254 thin-layer plates from Merck, and Amberlite XAD-4 from Rohm and Haas. The Amberlite was thoroughly cleaned by sequential extraction in a Soxhlet apparatus (Kontes) with chloroform, ethanol, and water for 24 h each. All evaporations were performed in a rotary evaporator at 35 °C.

RNA polymerase II was purified from raw wheat germ, obtained from General Mills, by the method of Jendrisak & Burgess (1975) and from calf thymus by the method of Schmincke & Hausen (1973). The standard assay mixture had a total volume of 0.25 mL and contained 2.5 μg of RNA polymerase II and final concentrations of 80 mM triethanolamine hydrochloride (pH 7.9 at 25 °C), 100 mM ammonium sulfate, 15% w/v glycerol, 1.5 mM $MnCl_2$, 0.1 mM disodium ethylenediaminetetraacetic acid, 5 mM thioglycerol, and 1 mM dithioerythritol. In all other aspects, the assay was performed as described by Jendrisak & Burgess (1975).

Identification of Toxins

At each step in the purification, an aliquot of the material was removed and stored at −20 °C. Toxins were located and identified by analytical thin-layer chromatography. Approximately 20 μg of toxin was spotted on a silica gel thin-layer plate together with authentic samples of phallacidin plus phallisacin, phalloidin, phalloin, and α-, β-, and γ-amanitin, all of which were kindly provided by H. Faulstich. The plate

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received January 9, 1978.* This work was supported in part by National Institutes of Health Grant No. AM 13230 to Jack L. Strominger. The author was supported by a National Science Foundation Predoctoral Fellowship followed by a National Institute of Medical Sciences Training Grant to the Biology Department of Harvard University.

0006-2960/78/0417-3786$01.00/0

was then developed in solvent I, 2-butanol:ethyl acetate: water:acetic acid (140:120:50:2), or solvent II, 2-butanol:3% ammonia (100:44; Faulstich et al., 1973). The plates were then dried and examined under short wave (254 nm) light to visualize ultraviolet absorbing compounds. The plates were then sprayed with 1% cinnamaldehyde in ethanol and suspended in a closed tank above concentrated HCl. In 15 min the amatoxins appeared as dark purple spots while the phallotoxins showed weaker blue spots.

Quantitation of Toxins

The total amount of amatoxin at each step in the purification was measured by the method of Preston et al. (1975). Fivefold serial dilutions of the sample in water were used to inhibit RNA polymerase II from calf thymus. Authentic β-amanitin was used as a standard. Stock solutions were made assuming $\epsilon = 16.4 \times 10^3$ cm^2/mmol at 303 nm (Preston et al., 1975).

Purification Procedure

A. *Mushroom Extract. Amanita phalloides* fruiting bodies were collected in New Jersey in the fall of 1975. Within 24 h, the mushrooms were air dried at 45 °C. The dried mushrooms were stored at room temperature in tightly closed plastic bags. In all subsequent steps disposable gloves were worn and all precautions appropriate for handling deadly poisons were observed. One hundred grams of dried mushrooms (equivalent to 830 g fresh) were soaked in 1500 mL of water for 1 min and then homogenized for 1 min at high speed in a 1-quart Waring blender. Ethanol (1500 mL of 95%) was added and the mixture was stirred for 4 h. The thick suspension was then filtered through Miracloth (Calbiochem). The material retained on the filter was added to 3 L of 95% ethanol plus 50 mL of acetic acid, stirred for 4 h, and filtered as before. The solid material was extracted a second time with 3 L of 95% ethanol and 50 mL of acetic acid. The filtrates of all three extractions were combined and evaporated. The resulting brown syrup was taken up in 100 mL of water, 200 mL of methanol, and 300 mL of chloroform and mixed thoroughly. The chloroform phase was separated and the aqueous layer was extracted twice with 100 mL of methanol plus 300 mL of chloroform. The combined chloroform phases were then back extracted twice with 100 mL of water plus 100 mL of methanol. The combined water layers, which contained the toxins, were filtered through Whatman 1 filter paper, evaporated, taken up in 250 mL of water, and refiltered.

B. *Desalting.* The 250 mL of aqueous phase from step A was loaded by reverse flow onto a 2.5 × 100 cm column of cleaned Amberlite XAD-4 equilibrated with water. After applying the sample, the column was washed with 1 L of water. The toxins, which adsorbed to the column, were recovered by removing the Amberlite from the column and batch eluting four times with a total of 2 L of 50% ethanol in a sintered-glass funnel. The combined eluates were evaporated.

C. *First Acidic LH-20 Chromatography.* The desalted mixture from step B was dissolved in 50 mL of water, adjusted to pH 3.0 with concentrated HCl, filtered through Whatman 1, and applied to a 4.0 × 100 cm column of Sephadex LH-20 equilibrated with 0.1 N acetic acid, pH 3. The sample was chromatographed in the same solvent at a flow rate of 100 mL per h. Fractions of 12.5 mL were collected and absorbance of the effluent was continuously monitored at 303 nm (the relative absorbance maximum for amatoxins) on a Gilford Model 2000 spectrophotometer equipped with a 2-mm flow cell. Two broad peaks of ultraviolet absorbing material eluted from the column. The first peak (fractions 35 to 116) contained brown hydrophilic material, but no toxins. The second (fractions 117–240)

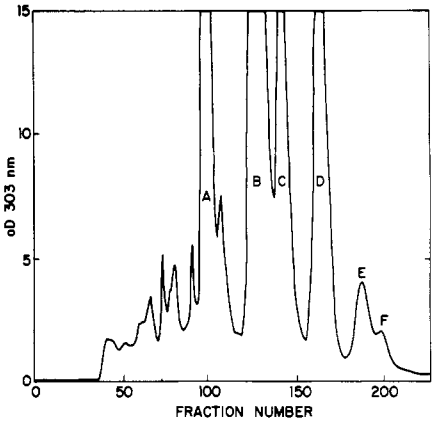

FIGURE 1: Neutral LH-20 chromatography. Material from step C was neutralized, evaporated, desalted, and chromatographed on a 2.5 × 200 cm column of Sephadex LH-20 at pH 6.5 as described in the text. Fractions were 10 mL and absorbance was measured at 303 nm. (A) Acidic phallotoxins; (B) β-amanitin; (C) phalloidin; (D) α-amanitin; (E) γ-amanitin; (F) phalloin.

contained the peptide toxins. These fractions were pooled, adjusted to pH 6.5 with ammonium hydroxide, evaporated, dissolved in 50 mL of water, and desalted on a 1.5 × 50 cm column of Amberlite XAD-4 as described above.

D. *Neutral LH-20 Chromatography.* The desalted mixture of toxins from step C was dissolved in 15 mL of 0.1 M ammonium acetate, pH 6.5, and chromatographed on a 2.5 × 200 cm column of Sephadex LH-20 in the same buffer at a flow rate of 50 mL per h. Fractions of 10 mL were collected and the effluent was again monitored at 303 nm (see Figure 1). Several minor peaks (fractions 35–100) were followed by six major peaks which contained the seven most abundant toxins. In order of elution, the peaks were: (A) phallacidin plus phallisacin; (B) β-amanitin; (C) phalloidin; (D) α-amanitin; (E) γ-amanitin; (F) phalloin. The peaks containing β-amanitin and phalloidin (fractions 121–154) were pooled and diluted by adding two volumes of water.

E. *QAE-Sephadex Chromatography.* QAE-Sephadex was converted to the acetate form by washing with 5 volumes of 1 N acetic acid followed by 5 volumes of water. The final slurry was adjusted to pH 6.5 with ammonium hydroxide and poured into a 1.5 × 50 cm column which was then washed with 5 volumes of water. The diluted solution from step D was then loaded onto the column at 200 mL per h. Fractions of 20 mL were collected and the effluent was monitored at 263 nm, the absorbance minimum for amanitins, in order to maximize the detection of impurities. The column was washed with water until the absorbance dropped and stabilized, and the adsorbed material was eluted with a 400-mL 0–0.5 M NaCl gradient. Phalloidin was found in the flow-through, β-amanitin eluted as a sharp peak at 0.2 M NaCl, and some brown material remained at the top of the column. Fractions 62–75 were pooled, desalted on Amberlite as before, and evaporated.

F. *Second Acidic LH-20 Chromatography.* The material at this point consisted largely of β-amanitin. The contaminants, as revealed by thin-layer chromatography, were some polar brown material and a second, less polar amatoxin, which was assumed to be ε-amanitin. Both contaminants were easily separated by dissolving the mixture from step E in 15 mL of 0.1 N acetic acid and chromatographing it on a 2.5 × 200 cm column of Sephadex LH-20 in the same solvent. The major ultraviolet absorbing peak, eluting between fractions 162 and 190, contained β-amanitin (see Figure 2). The pooled material was lyophilized.

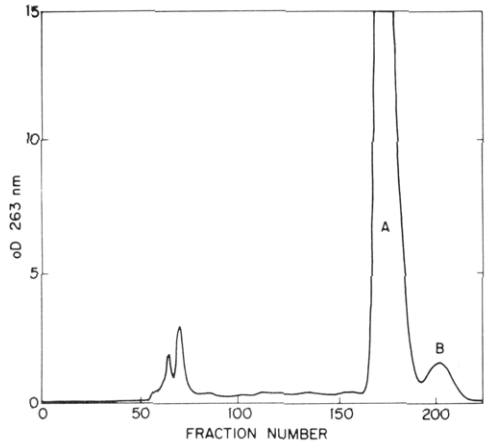

FIGURE 2: Second acidic LH-20 chromatography. Material from step E was evaporated, desalted, and applied to a 2.5 × 200 cm column of LH-20 at pH 3 as described in the text. Absorbance was measured at 263 nm and fractions were 10 mL. (A) β-Amanitin; (B) ε-amanitin.

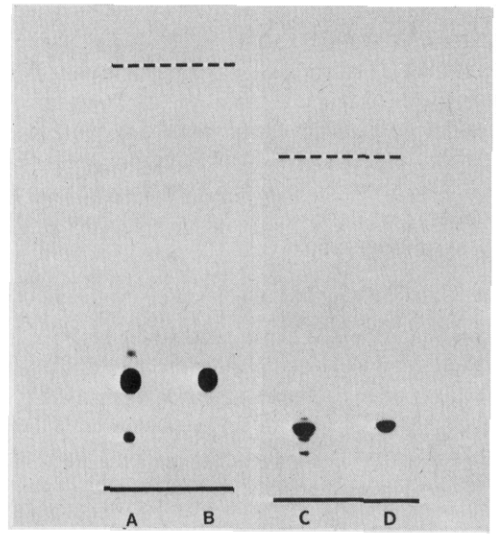

FIGURE 3: Thin-layer chromatography of purified β-amanitin. Fractions 162 to 190 from Figure 2 were pooled and lyophilized. Twenty micrograms was spotted on an analytical silica gel thin-layer plate beside 20 μg of an authentic reference compound and developed with solvent I or solvent II as described in the text. (A) Authentic β-amanitin; (B) β-amanitin from this study, solvent I; (C) authentic β-amanitin; (D) β-amanitin from this study, solvent II. The solid line represents the origin and the dashed line represents the solvent front.

G. *Purity*. The lyophilized fraction from step F was examined on thin-layer plates developed in solvent I or II. Both showed only one spot detectable by either ultraviolet fluorescence quenching or the cinnamaldehyde reagent. No additional spots were found by chromic acid charring or exposure to iodine vapors. This compound comigrated with authentic β-amanitin in both the acidic and the basic solvent systems (see Figure 3). When RNA polymerase II activity was tested against the purified poison under conditions described above, 50% inhibition occurred at 4.1×10^{-8} M for wheat germ enzyme and 6.1×10^{-9} M for the calf thymus enzyme. A summary of the purification is given in Table I.

TABLE I: Summary of Purification of β-Amanitin.[a]

purification stage	weight in g	β-amanitin equivalents in mg[b]	% yield
fresh mushrooms	830		
air dried	100		
crude extract	59	407	
chloroform extracted	48	398	
Amberlite XAD-4	6.1	369	
first acidic LH-20	1.6	375	
neutral LH-20	0.41	232	(100)
QAE-Sephadex	0.27	236	102
second acidic LH-20	0.24	216	93

[a] Yields could not be determined prior to the neutral LH-20 stage due to the multiplicity of amatoxins present. [b] Measured by inhibition of RNA polymerase II as described in the text.

Discussion

Fresh mushrooms undergo autolysis and do not keep well. Furthermore, there is a report of an "amanitinase" that is active in methanol extracts of fresh toadstools, but inactive in similar extracts of dried tissue (Palyza, 1974). Therefore dried carpophores were used in this study. The toxins appeared to be perfectly stable to air drying and storage is convenient. Ethanol proved to be adequate for completely extracting the toxins from the dried material. After extraction as described above, no additional quantities of the toxins could be dissolved from the pulp with water or methanol.

The ethanol extract contained large amounts of inorganic salts, saccharides, nucleotides, lipids, and the peptide toxins. The lipids were effectively removed by the chloroform extraction. The problem of removing the salts from the crude material as well as during later stages of the purification was of central importance because high concentrations of salt interfere strongly with Sephadex chromatography by causing the swollen beads to collapse and also by altering the mobilities of the peptide toxins. Amberlite XAD-4, which is a macroporous polystyrene resin with no ion-exchange groups, adsorbed both families of toxins quantitatively from aqueous solutions, presumably by a hydrophobic interaction. Inorganic salts were not retained and were removed in the wash. This allowed for an "instant dialysis" of the toxins. The XAD-4 also afforded a substantial purification by passing much of the polar brown material and saccharides in the crude extract and by retaining some of the nonpolar material in 50% ethanol. The ethanol elution could not be carried out in the column because of the unavoidable formation of bubbles due to the lower solubility of gases in 50% ethanol and the heat which evolved when the 50% ethanol and water mixed. For this reason the batch elution method was chosen. This single step replaces multiple precipitations in cold methanol, lead acetate precipitation, ammonium sulfate precipitation, isopropyl alcohol extraction, and further cold methanol precipitation in the previously published method (Wieland, 1967).

The use of Sephadex LH-20 for resolution of the individual toxins was based on the observation by Faulstich et al. (1973) that the major toxins are well separated on this hydrophobic gel on an analytical scale. An important feature of LH-20 was that the elution volume of the peptides was proportional to their hydrophobicity. Thus by changing the pH of the buffer from 6.5 to 3 and thereby suppressing ionization of the aspartic acid residue, the mobility of β-amanitin of LH-20 was shifted from 1.29 to 1.70 column volumes. The acidic phallotoxins behaved similarly. This was advantageous because the mobilities of many unwanted compounds shifted in the opposite direction or remained the same. Hence the bulk of the brown polar material remaining after the desalting step could be separated from the toxins on a short, fat, low resolution column, which then allowed separation of the individual toxic components on

a long, thin, high resolution column of neutral LH-20. It is crucial to point out that different lots of LH-20 varied with respect to the mobilities of the peptide toxins. For example, Sigma lot no. 105C-0060 gave an entirely different pattern from that given by lot no. 104C-0023, the one used in the study described here. Thus, at least one batch was not useful for separating these toxins.

Although β-amanitin and phalloidin were not entirely separated on neutral LH-20, they were easily separated on QAE-Sephadex. β-Amanitin bound at low ionic strength due to its negative charge, while phalloidin, being uncharged, flowed through the column.

The apparent loss of activity on the second acidic LH-20 column was in part due to the removal of ε-amanitin, which is also active in inhibiting RNA polymerase II (see Figure 2 and Table I). The discrepancies between β-amanitin equivalents and actual weights in Table I were probably due to incomplete dehydration of the samples.

Throughout the purification, the pH of all solutions was maintained between 3 and 6.5. Below pH 3, the γ-hydroxyisoleucines cyclize to the lactone with concomitant opening of the peptide ring and loss of activity (Wieland & Wieland, 1972). Therefore solutions of toxins in 0.1 N acetic acid must be neutralized before rotary evaporation or else lyophilized. Otherwise the acetic acid concentration increases and the polar decomposition product appears. Above pH 9, the 6-hydroxyl group of the tryptophan ionizes, and a rearrangement occurs at the sulfoxide to give an inactive product (Faulstich et al., 1974). For this reason an alkali extraction as described by Faulstich et al. (1973) was avoided.

The final product of this purification was pure as judged by thin-layer chromatography using the four methods of detection described above. The overall yield of 2.16 mg/g dry weight of dried mushrooms is close to the 2.3 mg/g dry weight found in these mushrooms in an analytical study (Yocum & Simons, 1977). The purified β-amanitin inhibited calf thymus RNA polymerase II at a concentration similar to that previously reported (Faulstich et al., 1974).

The β-amanitin obtained in this study produced crystals which were large enough to permit determination of its three-dimensional structure by X-ray diffraction. This analysis is reported in the accompanying paper (Kostansek et al., 1978).

Acknowledgments

The author would like to thank Jack Strominger for supporting this research, Josiah Wedgwood, Cox Terhorst and Charles Yocum for essential discussions, Robert Sauer and Donald Simons for critically reading the manuscript, Donald Simons and C. Moore for help in collecting mushrooms and T. Wieland and H. Faulstich for samples of authentic toxins.

References

Cochet-Meilhac, M., & Chambon, P. (1974) *Biochim. Biophys. Acta 353,* 160.

Faulstich, H., Georgopoulos, M., & Bloching, M. (1973) *J. Chromatogr. 79,* 257.

Faulstich, H., Trischmann, H., & Wieland, T. (1974) in *Peptides 1974* (Wolman, Y., Ed.) pp 333–338, Halsted Press, New York, N.Y.

Faulstich, H., Brodner, S., Walch, S., Wieland, T. (1975) *Justus Liebigs Ann. Chem.,* 2324.

Jendrisak, J., & Burgess, R. (1975) *Biochemistry 14,* 4639.

Kostansek, E., Yocum, R., Thiessen, W., & Lipscomb, W. (1978) *Biochemistry 17* (following paper in this issue).

Lengsfeld, A., Löw, I., Wieland, T., Dancker, P., & Hasselbach, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2803.

Lynen, F., & Wieland, U. (1937) *Justus Liebigs Ann. Chem. 533,* 93.

Palyza, V. (1974) *Arch. Toxicol. 32,* 109.

Preston, J., Stark, H., & Kimbrough, J. (1975) *Lloydia 38,* 153.

Schmincke, C., & Hausen, P. (1973) *Z. Naturforsch. C 28,* 610.

Wieland, T. (1967) *Fortschr. Chem. Org. Naturst. 25,* 214.

Wieland, T., & Wieland, O. (1972) in *Microbial Toxins, Vol. 8* (Kadis, S., Cigler, A., & Ajl, S., Eds.) pp 249–280, Academic Press, New York, N.Y.

Wieland, T., Wirth, L., & Fischer, E. (1949) *Justus Liebigs Ann. Chem. 564,* 152.

Wieland, T., Mannes, K., & Schöpf, A. (1958), *Justus Liebigs Ann. Chem. 617,* 152.

Wieland, T., Rempel, D., Gebert, U., Buku, A., & Boehringer, H. (1967) *Justus Liebigs Ann. Chem. 704,* 226.

Yocum, R., & Simons, D. (1977) *Lloydia 40,* 178.