Journal of Chromatography, 462 (1989) 442–447
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 008

Note

Assay for the main phallotoxins in *Amanita phalloides* Fr. by direct fluorimetry on thin-layer plates

F. ENJALBERT*

Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Avenue Charles Flahaut, 34060 Montpellier Cedex (France)

M. J. BOURRIER

Laboratoire Interrégional de la Répression des Fraudes, 2 Rue Saint-Pierre, 34024 Montpellier Cedex (France)

and

C. ANDARY

Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie, Avenue Charles Flahaut, 34060 Montpellier Cedex (France)

(First received May 16th, 1988; revised manuscript received September 23rd, 1988)

As phallotoxins are not taken up by intestinal epithelial cells, they do not play a role in poisoning by the death cap mushroom. The toxic effects after eating the mushroom are caused solely by amatoxins[1,2]. However, like amatoxins, phallotoxins have become important tools in biochemical and biological research. They inhibit the conversion of actin F into actin G and disturb the dynamic equilibrium of these forms which are necessary in cell functions[3]. In addition, when taken up selectively by hepatocytes, they cause cholestasis by interaction with the polymerization of hepatocellular actin and are used as hepatotoxic substances[4,5].

Phalloidin was the first phallotoxin isolated from *Amanita phalloides*. Other toxins derived from this structure were then characterized. Phallotoxins are dicyclic heptapeptides which are divided into two groups. The first group consists of neutral molecules: phalloin (PHN), phalloidin (PHD), phallisin (PHS) and prophalloin (PPN). The second consists of the acid molecules phallacin (PCN), phallacidin (PCD) and phallisacin (PSC). The presence of an hydroxyl group on proline makes these molecules toxic. The LD_{50} of PPN, for example, which has lost this OH, is ten times as great[6].

Knowledge of the locations of phallotoxins in the mushroom was hindered by the lack of sufficiently sensitive and reliable methods of detection. The isolation techniques proposed use thin-layer or column chromatography. After separation of the toxins, identification was carried out using UV absorption spectophotometry or colorimetric reactions[7–9]. High-performance liquid chromatography (HPLC) was applied mainly to biological liquids[10]. Assaying was carried out using chemical or biological methods. The first methods include the evaluation of gammalactones derived from amino acids resulting from the hydrolysis of toxins and separated by column chromatography[7] and fluorimetric detection of cyclopeptides before and after hydro-

0021-9673/89/$03.50

lysis[11]. Biological techniques make use of the affinity of phallotoxins for muscle actin which is a target protein. One biological assay uses the polymerizing effect of [^{3}H]dimethylphalloin on rabbit muscle actin[12]. Another more sensitive assay is based on the protective effect of PHD on the inhibition of a pancreatic desoxyribonuclease by actin[13]. Biological assaying is of great value since its high sensitivity (detection limit 6.3 ng) has made it possible to detect phallotoxins in other species of the genus *Amanita* such as *A. vera, A. subalbiacea, A. mutabilis* and *A. rubescens*[13].

However, these biological techniques result in an overall assay of these molecules and are thus ineffective in a search for the locations of the various neutral and acid phallotoxins in the mushroom. A sensitive, reproducible assaying technique is therefore proposed for the main phallotoxins present in *A. phalloides*. It was used to determine these toxins in each part of the mushroom (cap, stipe and volva).

MATERIALS AND METHODS

Extraction

A fragment of each organ of fresh mushroom (0.5 to 1.5 g) was crushed and extracted with methanol–water–0.01 *M* hydrochloric acid (5:4:1). Three extractions were carried out ultrasonically at 40°C using 7 ml of extraction medium for each extraction. The samples were rinsed twice in 5 ml of solvent, pooled, filtered and concentrated in a rotary evaporator at 35°C until a dry residue was obtained. This was then resuspended in methanol–water (8:2) to obtain a 20% extraction solution for the assays.

Thin-layer chromatography (TLC)

The following materials and reagents were employed: silica gel plates. Ref. 5721 (Merck, Darmstadt, F.R.G.); "Gold label" microcaps (Drummond, Broomal, PA, U.S.A.); calibration solution of phalloidin (Boehringer, Mannheim, F.R.G.); $1 \cdot 10^{-4}$g/ml in methanol–water (10:90); tank lined with filter-paper at room temperature (20 ± 2°C); solvent chloroform–methanol–28% ammonia (60:40:10).

F-detection two solutions were used: (a) 2.5% (w/v) 4-dimethylaminobenzaldehyde (PDAB) (Fluka, Buchs, Switzerland) in methanol; (b) 2 ml of concentrated sulphuric acid (Prolabo, Paris, France) added gradually while shaking to 10 ml of methanol–glycerol (6:4). An UV chromatographic analyser, Farrand Vis 2 (Optical, New York, NY, U.S.A.) with two monochromators, operating by fluorescence and by reflection (excitation at 325 nm, emission at 480 nm) was employed.

Procedure

A 15- to 20-μl volume of 20% mushroom extract and 0.5, 1, 3, 5 and 7 μl of phalloidin solution, corresponding to 50, 100, 300, 500 and 700 ng of toxin were placed on a silica gel plate with a micropipette. The plate was placed in the tank, which was saturated with solvent vapour, and developed to an height of 16 cm (duration 100 min). After drying under cold air to remove the alkaline residue and traces of developing solvent, the chromatogram was visualized by spraying with solution (a) followed by drying. This operation was carried out three times. Solution (b) was then sprayed on to the plate which was subsequently dried in an oven at 115°C for 10 min. After removal from the oven the chromatogram was covered with a glass plate until it was completely cold.

Phallotoxins were visible as white fluorescent spots after exposure to UV light at 360 nm. Levels were determined by referring to a calibration line representing the variation of peak area of the PHD standard as a function of concentration.

RESULTS AND DISCUSSION

Extraction

The extraction method was chosen in accordance with the literature. Most of the tests were carried out with alcohol: ethanol or methanol[14] or a mixture of alcohol and water (1:1)[8,9] or methanol–ammonia[7]. Methanol–water (1:1) treatment was effective, although methanol–water–0.01 *M* hydrochloric acid (5:4:1) was preferred as the yield of PHD was greater. A slightly acid pH appeared to enhance the stability of the toxins[8]. Most authors[7–9,11] then purified the extracts; this involved long operations which inevitably resulted in loss of toxins. This was avoided in the present work by the use of raw extracts obtained using this simple and reproducible extraction method.

The advantage of carrying out these tests on fresh material should also be noted. Drying causes considerable degradation of phallotoxins, which are comparatively thermolabile molecules. Up to 30% loss of toxins was observed in assays carried out in parallel on fresh material and dried material from the same cap.

The reproducibility of this method of extraction was verified by carrying out several assays on extracts from the same organ. The average deviation between the levels of the various toxins was of the order of 3%.

Assays

Solvents. Several eluting agents recorded in the literature were tested[7,8,15,16]. One of them[16] gave good separation of the neutral toxin group (PHN, PHD, PHS) but the gap between the R_F values of PCD and PSC was too small to permit photodensimetric assay.

A more polar solvent was therefore devised: chloroform–methanol–28% ammonia (60:40:10). This gave better separation of the five phallotoxins from each other and from other spots. Chromatograms with well defined, well separated spots perfectly suitable for photodensimetric assay were obtained. The mean R_F values of the main phallotoxins in this solvent are shown in Table I.

TABLE I

MEAN R_F VALUES IN SILICA GEL TLC OF THE MAIN PHALLOTOXINS IN *AMANITA PHALLOIDES*

Solvent: chloroform–methanol–28% ammonia (60:40:10).

Phallotoxin	R_F *(mean ± S.E.M., n = 3)*
PHN	0.60 ± 0.03
PHD	0.50 ± 0.02
PHS	0.44 ± 0.03
PCD	0.21 ± 0.02
PSC	0.14 ± 0.01

Detection. Sensitive, stable resolution was desired which would enable direct assay on a chromatoplate. A fluorimetric detection method was used first with phosphoric acid at 80°C recommended for resolution of α-amanitin[17]. This led to several trials with different acids: sulphuric acid, perchloric acid and trichloroacetic acid at between 80 and 100°C. Sulphuric, phosphoric and perchloric acids reacted with approximately the same sensitivity but the fluorescence was fugacious. In order to improve this medium sensitivity, we tested the addition of several aldehydes (cinnamaldehyde, anisaldehyde, *p*-dimethylaminobenzaldehyde and *p*-dimethylaminocinnamaldehyde). A mixture of PDAB and sulphuric acid gave better sensitivity but the reaction was not sufficiently stable for assays. The stability of fluorescence was considerably improved by the addition of glycerol to the acid mixture. In this procedure the reagent was used in two steps and the chromatogram was protected with a glass plate until it had cooled completely; this made it possible to fix the reaction. Phallotoxin fluorescence resolved in this way remained stable for several hours and permitted fluorodensimetric assaying with a sensitivity of 25 ng per spot on the chromatograms.

Sensitivity, validation and precision

The resolution method generally used is based on the colorimetric reaction produced by contact of cinnamic aldehyde with hydrochloric acid vapour. Phallotoxins appeared on the chromatogram in the form of blue-grey spots and disappeared very rapidly. The limit of detection was of the order to 500 ng per spot[6]. The resolution was 20 times as sensitive when the PDAB–sulphuric acid based reagent was used. The results reported in Fig. 1 indicate that the validation of the method is satisfactory

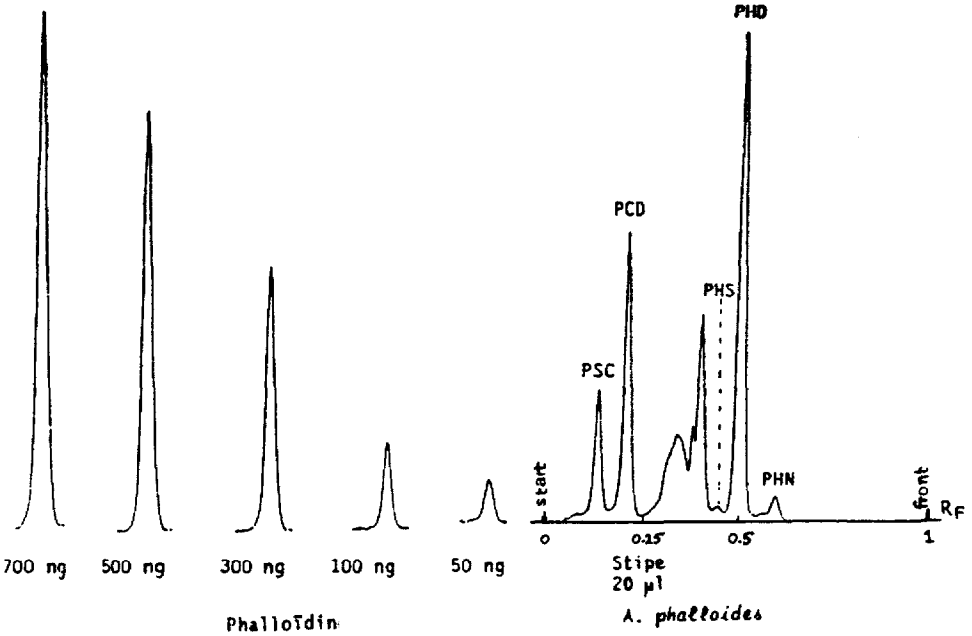

Fig. 1. Graphic plotting after fluorodensitometric reading (excitation at 325 nm, emission at 480 nm) of the spots in silica gel TLC of a calibration range of phalloidin and an extract of *Amanita phalloides* stipe.

since the calibration graph for PHD is linear from 50 to 700 ng (coefficient of correlation, $r = 0.9996$). The regression equation indicating the peak area (mm^2) *versus* the PHD concentration is as follows: $s = 0.223[PHD] - 2.11$, with standard deviations of slope and intercept of 0.004 and 1.42 respectively. The quantification limit is 8 μg of toxin per g of fresh material. The inter-assay precision was verified by subjecting the same extract (accompanied in each case by the same series of standard solutions) to three assays (Table II). The coefficient of variation (standard deviation/mean) calculated for PHD, PCD and PSC was found to be 5%. It was not calculated for PHN since the values were too small.

The technique recommended can be used to measure the amounts of five phallotoxins in the various organs (cap, stipe and volva) of an *Amanita phalloides*. PHN, PHD, PHS, PCD and phallisacin PSC were characterized using the reference substances kindly provided by Professor H. Faulstich. In the absence of a PCN standard, it was not possible formally to locate and assay this acid molecule on the chromatograms. However, it is noted that in previous work PCN was found only in very small amounts in a single specimen (less than 1/30 of PCD content)[18]. In addition, the low toxicity of PPN led to its exclusion from the study[6].

For practical reasons, all assays are expressed in mg of PHD per g of fresh organ. Indeed, phalloidin (commercially available) was the only pure toxin in our possession.

Fig. 1 shows the graphic plotting of the chromatographic spots of an organ (stipe) extract of *A. phalloides* prepared using the method described together with the calibration range. As the plot is linear from 50 to 700 ng, it was possible to assay the five toxins using the same preparation. Table II shows the amounts of each phallotoxin and total (T) in each organ (cap, stipe and volva); the last column gives the concentrations for the whole mushroom calculated from the respective weights of the three organs.

According to these results, the volva is the organ richest in phallotoxins and the stipe is the poorest. These data are in perfect agreement with Faulstich's observa-

TABLE II

MEAN PHALLOTOXIN CONTENT IN mg/g OF FRESH SPECIMEN IN THE VARIOUS ORGANS OF A SINGLE SPECIMEN OF *AMANITA PHALLOIDES* (MEAN ± S.E.M., $n = 3$) AND PHALLOTOXIN CONTENT OF THE WHOLE MUSHROOM CALCULATED USING THE RESPECTIVE WEIGHTS OF THE THREE ORGANS

C = cap; S = stipe; V = volva; W = whole fungus. T = All toxins. S.E.M. values of PHN and PSC are corrected to three decimal places.

Toxins	*C* *(30.073 g)*	*S* *(11.1 g)*	*V* *(1.006 g)*	*W* *(42.179 g)*
Neutral				
PHN	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.008
PHD	0.141 ± 0.004	0.114 ± 0.003	0.275 ± 0.008	0.137
PHS		Traces		
Acidic				
PCD	0.059 ± 0.002	0.055 ± 0.002	0.069 ± 0.002	0.056
PSC	0.022 ± 0.001	0.022 ± 0.001	0.024 ± 0.001	0.022
T	0.298 ± 0.004	0.275 ± 0.004	0.448 ± 0.008	0.295

tions[6]. With regard to the distribution of toxins in each organ, it will be noted that the major toxin was PHD, followed by PCD and PSC. The amounts of PHN were very small and traces of PHS were found only in the stipe. PHD and PCD were thus the main toxins of the neutral and acid phallotoxin groups respectively. This was also a feature of the assays carried out previously by other authors[8,9,18]. However, the contents of each of the two molecules are very different to those in the literature; our results show that PHD was predominant whereas the dominance of PCD had always been noted[8,9,18].

The rapidity of the method means that it can be applied to a large number of specimens to measure the variations in the levels of these toxins as a function of the ecology and age of the fungus.

ACKNOWLEDGEMENT

The authors thank Professor H. Faulstich for kindly providing phallotoxin standards not commercially available: phalloin, phallisin, phallacidin and phallisacin.

REFERENCES

1 H. Faulstich and K. Munter, *Klin. Wochenschr.*, 64, suppl. VII (1986) 66.
2 T. Wieland, *Naturwissenschaften*, 74 (1987) 367.
3 T. Wieland, *Int. J. Pept. Protein Res.*, 22 (1983) 257.
4 H. Faulstich, K. Munter and D. Mayer, *Falk Symp.*, 38 (1984) 183.
5 M. Frimmer, *Toxicol. Lett.*, 35 (1987) 169.
6 T. Wieland and H. Faulstich, in R. F. Keller and A. T. Tu (Editors), *Handbook of Natural Toxins*, Vol. I, Marcel Dekker, New York, 1983, Ch. 18, p. 585.
7 H. Faulstich, D. Georgopoulos and M. Bloching, *J. Chromatogr.*, 79 (1973) 257.
8 R. R. Yocum and D. M. Simons, *Lloydia*, 40 (1977) 178.
9 J. A. Beutler and A. H. Der Marderosian, *J. Natl. Prod.*, 44 (1981) 422.
10 G. Caccialanza, C. Candini and R. Ponci. *J. Pharm. Biomed. Anal.*, 3 (1985) 179.
11 M. C. Little and J. F. Preston, *J. Natl. Prod.*, 47 (1984) 93.
12 J. A. Schäfer and H. Faulstich, *Anal. Biochem.*, 83 (1977) 720.
13 J. E. Mullersman and J. F. Preston, *Anal. Biochem.*, 119 (1982) 266.
14 T. Stijve and R. Seeger, *Z. Naturforsch.*, 34C (1979) 1133.
15 C. Andary, F. Enjalbert, G. Privat and B. Mandrou, *J. Chromatogr.*, 132 (1977) 525.
16 S. H. A. Malak, *Planta Med.*, 29 (1976) 80.
17 Y. Plancke, J. P. Henichart and J. L. Bernier, *Z. Naturforsch.*, 35C (1980) 516.
18 H. Faulstich, D. Georgopoulos, M. Bloching and T. Wieland, *Z. Naturforsch.*, 29C (1974) 86.