CEREAL CHEMISTRY

VOL. 44 JANUARY, 1967 No. 1

A METHOD FOR THE DETECTION AND ESTIMATION OF AFLATOXIN IN FUNGAL FERMENTATION PRODUCTS[1]

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ABSTRACT

A procedure representing a rapid method for determining the possible presence of aflatoxin in fungal fermentation products such as enzyme preparations has been evaluated. Thin-layer chromatography on silica gel of the chloroform extracts from seven different commercial fungal enzymes all showed a fluorescing spot having an R_f higher than aflatoxin B_1. The ultraviolet and infrared spectra of this component differ from those of aflatoxin B_1. This component also differs from aflatoxin B_1 in that it does not give a fluorescing derivative with formic acid in the presence of thionyl chloride. The data show that aflatoxin is not present in any of the fungal enzyme preparations tested.

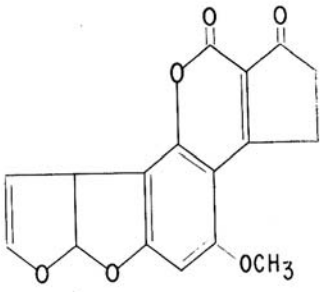
Some strains of *Aspergillus flavus* and related aspergilli elaborate aflatoxin under certain conditions. Because of the histopathological effects of aflatoxin, including carcinogenic activity on the livers of some test animals, the determination of its presence in foods and feeds, and particularly in peanut products, has become a matter of concern to many food producers and regulatory agencies. Because of possible aflatoxin hazard, a study was undertaken 1) to demonstrate that aflatoxin is not present in the commercial enzymes produced by *Aspergillus* fungal fermentations and widely used by the food industry, and 2) to provide a satisfactory analytical method for determining aflatoxin in products such as enzymes derived from fungal fermentations.

Aflatoxin is commonly a mixture of four components whose chemical structures and relationships have been determined (1–4). Since aflatoxin B_1 is the most prevalent and also the most toxic, a measure of this component is usually taken as the basis for a quantitative estimate of product toxicity. The structure of aflatoxin B_1 is given in Fig. 1.

A number of methods have been described for determining aflatoxin in peanuts and peanut products. Most of the published methods were developed for food products and are more complicated than is desirable for routine use, involving the use of columns for separation of the components extracted with the various solvents (5–7). Furthermore, these methods do not lend themselves readily to use with enzyme products, since methanol, hexane, and acetone, the most used solvents, extract rather large amounts of solids which in the case of enzymes contain considerable quantities of interfering fluorescing materials.

Of the methods surveyed, the method of W. V. Lee (8) is by far the

[1]Manuscript received May 12, 1966. Presented at the 51st annual meeting, New York City, April 1966. Contribution from the Enzymology Research Laboratory, Miles Laboratories, Inc., Chemicals Division, Elkhart, Ind.



AFLATOXIN B_1

Fig. 1. Aflatoxin B_1.

simplest, involving extraction by chloroform in the presence of water at laboratory temperatures, and was the procedure used in our studies.

The absence of any tolerances for aflatoxins makes proper selection of sample size extremely important. Results of a market survey on a number of foods which use fungal enzymes during processing indicated that the average daily intake of enzyme was slightly under 0.01 g. per day. One hundred times this amount or 1.0 g. was used for extraction in most of the work reported in this paper.

Materials and Methods

In this study seven enzyme preparations from fungal sources were used. These are listed below. Though only one genus and three different species are

Enzyme	*Source Organism*	*Enzyme*	*Source Organism*
Amylase (type 1)	*A. oryzae*	Pectinase	*A. niger*
Amylase (type 2)	*A. oryzae*	Cellulase	*A. niger*
Amyloglucosidase	*A. foetidus*	Hemicellulase	*A. niger*
Glucose oxidase	*A. niger*		

listed, the enzymes represent seven different strains and seven different fermentation processes. Amylase type 1 is distinguished from type 2 in that it produces considerably more maltose and less dextrose from starch.

The extraction and estimation procedure used in these studies was as follows.

1. One gram of enzyme was weighed into a 50-ml. glass-stoppered flask.

2. Just enough water to wet the enzyme was added. This varied from 1.0 to 2.5 ml. depending on the enzyme.

3. Ten ml. of chloroform was then added and the mixture was shaken vigorously for 3 min.

4. Ten ml. additional chloroform was added and the mixture was shaken for 1 min.

5. The flask was then placed on a rotary shaker for 30 min.

6. After shaking, the contents of the flask were transferred to a separatory funnel. The flask was washed with 5 ml. of chloroform and the wash also was transferred to the separatory funnel.

7. When the chloroform phase separated from the brown aqueous layer, it was drawn off into a beaker. The aqueous phase was washed with 10 ml. of chloroform and the chloroform phase was again drawn off and combined with the first chloroform extract.

8. The chloroform extract was then evaporated to about 20 ml. and transferred to a small vial, where it was evaporated to dryness.

9. The residue was taken up in 200 μl. of chloroform, and 20 μl. was spotted on Eastman Chromogram silica gel thin-layer sheets (type K301R2) and developed with 3% of methanol in chloroform until a solvent path-length of 12–13 cm. from the base line was obtained.

10. The dry, developed chromatoplate was then examined under long-wave ultraviolet light for the presence or absence of purple-blue fluorescent spot of R_f value 0.5 to 0.55 corresponding to aflatoxin B_1 and verified by spotting a standard solution of aflatoxin B on the same plate for comparison.

11. Since on our plates 0.005 γ of aflatoxin B_1 was plainly visible, the absence of a spot would indicate a concentration of less than 0.05 p.p.m. Actually 0.002 γ could be seen, but we did not feel the spot was intense enough to be picked up in the presence of traces of interfering material.

Coomes *et al.* (6) claim a sensitivity of 0.0004 γ for aflatoxin B_1 on Kieselgel G (508 $\pm$ 10 μ thick). With this sensitivity the absence of a blue fluorescent spot would indicate an aflatoxin B_1 concentration of less than 0.004 p.p.m. or 4.0 p.p.b.

Results and Discussion

To test the efficacy of the method, known amounts of aflatoxin were added to 1-g. portions of an enzyme preparation, and the extraction was carried out as described. When the chloroform extract had been taken to dryness, the residue was dissolved in 5 ml. of chloroform and the absorbance was read in a Beckman DU Spectrophotometer at 360 mμ. As indicated by the data in Table I, recovery was complete in all cases.

TABLE I
RECOVERY BY CHLOROFORM EXTRACTION OF AFLATOXIN ADDED TO AN ENZYME PREPARATION

SAMPLE	AFLATOXIN ADDED	Absorption		
		at 360 mμ Extract	Corr.	Theory
	mg.			
Control	0	0.130	0	0
1	1	0.147	0.017	0.012
2	5	0.184	0.054	0.053
3	20	0.342	0.212	0.212

With the efficacy of the method established, the next step was to carry out extractions on the various fungal enzymes to see if aflatoxin was present. The chloroform extracts were taken to dryness in tared vials and the residue was weighed; the table below shows the solids removed. Of interest is the fact that

Enzyme	*Residue*	*Enzyme*	*Residue*
	%		%
Amylase (type 1)	0.17	Glucose oxidase	0.03
Amylase (type 2)	0.04	Cellulase	0.45
Pectinase	0.03	Hemicellulase	0.03
Amyloglucosidase	0.07		

extraction of cellulase gave a much heavier residue than the other enzymes. This manifests itself in the fact that cellulase was the only enzyme which tended to form an emulsion during extraction. However, vigorous shaking in the separatory funnel or centrifugation usually brought about separation of the chloroform phase.

The extracts were taken up in chloroform and their absorption spectra were determined on a Beckman DU from 250 to 450 mμ. The absorption spectrum of aflatoxin was also obtained in the same manner. Figure 2 gives the results of these experiments. Absorbance was plotted on the basis of each enzyme extract being dissolved in 5 ml. of chloroform. It can be seen that the shapes of the curves are similar with absorption maxima around 280 mμ. The absorbances, moreover, are proportional to the weight of residue extracted, indicating similar composition. Indeed, when infrared spectra were run on 1-mg.

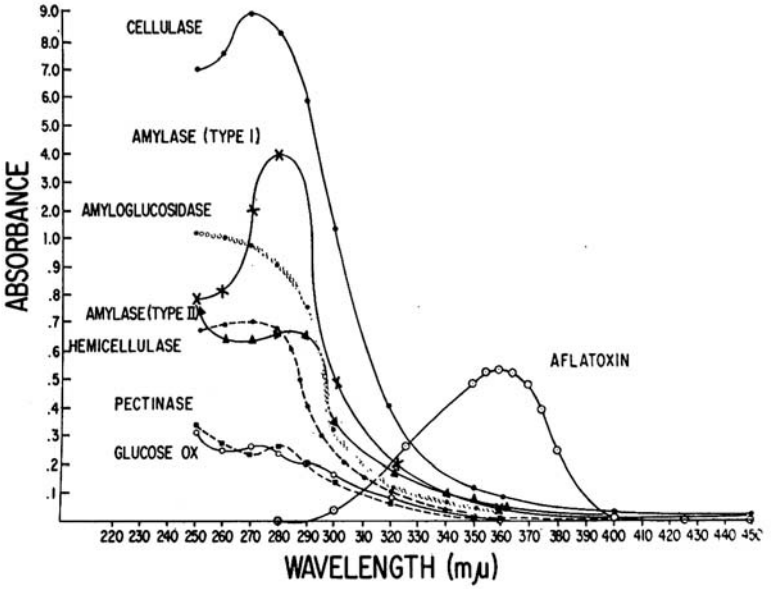


Fig. 2. Absorption spectra of chloroform extracts of various fungal enzyme preparations and of aflatoxin.

samples of extracts from cellulase, amyloglucosidase, and pectinase, they were identical except for pectinase, which showed slight variation in pattern between 6.25 and 7.25 μ.

Further observation of the ultraviolet spectra shows all of the curves to be quite different from that of aflatoxin, and none of the extracts show a maximum at 360 mμ, characteristic of the aflatoxin. This, however, does not rule out the possibility of traces of aflatoxin being present, since ultraviolet absorption is much less sensitive than fluorescence.

To further characterize these extracts, 1-g. samples of the enzymes were treated with chloroform, and the chloroform extracts were evaporated to dryness. Each residue was taken up in 200 μl. of chloroform, and 20-μl. aliquots were spotted on silica gel thin-layer plates. After development with 3% methanol in chloroform, the chromatoplate was viewed under a Kensco ultraviolet lamp at both short and long wave length. As can be seen from Fig. 3, all the enzymes contained a common major component. This component had an R_f of 0.615 compared to 0.50 for aflatoxin B_1. It also displayed a white fluorescence instead of the purple-blue displayed by aflatoxin. Further, the fluorescence was more intense under short-wave ultraviolet than long-wave ultraviolet.

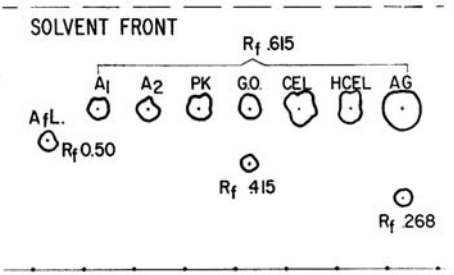


Fig. 3. Thin-layer chromatogram of aflatoxin and of chloroform extracts of fungal enzymes. Solvent: 3% methanol in chloroform.

Two of the enzymes, glucose oxidase and amyloglucosidase, showed second spots. The second spot from the glucose oxidase extract had an R_f of 0.415; that from amyloglucosidase had an R_f of 0.268. Both showed white fluorescence which was more intense under short-wave than long-wave ultraviolet.

As a further check, the remainder of each of the extracts (180 μl.) was spotted on Whatmann No. 3 Paper (18¼ × 22½ in.) and developed with 3% methanol in chloroform. In each case only the fast-moving common component was observed. There was no tailing, and no other spots were found.

To further establish the nonidentity with aflatoxin B_1 of the component extracted from the enzymes, the following experiment was run. One gram of enzyme was extracted with chloroform as described in the procedure. To a second 1-g. quantity, 0.4 γ of aflatoxin was added and this was then extracted. The two extracts were each taken up in 200 μl. of chloroform and 20 μl. of

each was spotted on a thin-layer chromatoplate along with 0.05 γ of standard aflatoxin. The remainders of the two extracts were taken just to dryness and then dissolved in 100 μl. of chloroform. To each of these samples 0.2 ml. of formic acid and 1 drop of high-grade thionyl chloride were added according to the procedure described by Andrellos and Reid (10). The tubes were stoppered and the contents shaken until homogeneous. The mixtures were let stand for 5 min. at room temperature and then evaporated just to dryness. The residues were dissolved in 50 μl. of chloroform, and 20-μl. aliquots were spotted on the thin-layer chromatoplate. After development with 3% methanol in chloroform, the chromatoplate was viewed under ultraviolet light. The results are shown in Fig. 4. Reading left to right, the spots are standard aflatoxin B_1, formate derivative of aflatoxin, enzyme extract, the formate derivative of the enzyme extract, the extract of the enzyme plus aflatoxin, and the formate derivative of the enzyme plus aflatoxin extract. One can see from this chromatogram that the enzyme extract alone did not form a formate derivative and, in the case of the enzyme plus aflatoxin extract only the aflatoxin formed a formate derivative.

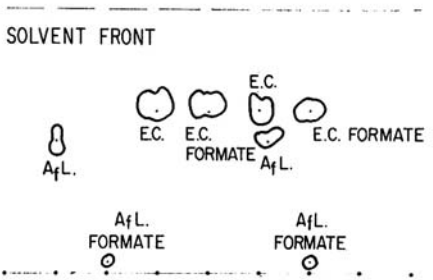


Fig. 4. Thin-layer chromatogram of aflatoxin and of component from chloroform extract of enzyme and their formate derivatives.

Finally, we extracted about 10 g. of enzyme and spotted numerous aliquots on thin layer. After development as usual we eluted the spots into chloroform, filtered off the silica gel, evaporated the eluate to dryness, and ran an infrared spectrum on this component. Figure 5 shows the spectrum of the enzyme component compared to that of aflatoxin B_1. Visual inspection shows the two compounds to be quite different. The enzyme component shows a peak at about 3.4 μ representing a CH_2 chain which is absent in aflatoxins. Further, aflatoxin shows a peak at 5.65–5.69 μ, suggesting a lactone grouping which is not present in the spectrum of the enzyme component.

In summary, this procedure offers a simple, rapid method for determining the possible presence of aflatoxin B_1 in fungal enzymes. The sensitivity in our hands was about 50 p.p.b. However, according to the literature, with Silica Gel G-HR one can see 0.0004 γ of aflatoxin B_1, which would increase the sensitivity to about 4 p.p.b. The use of paper chromatography, though increasing

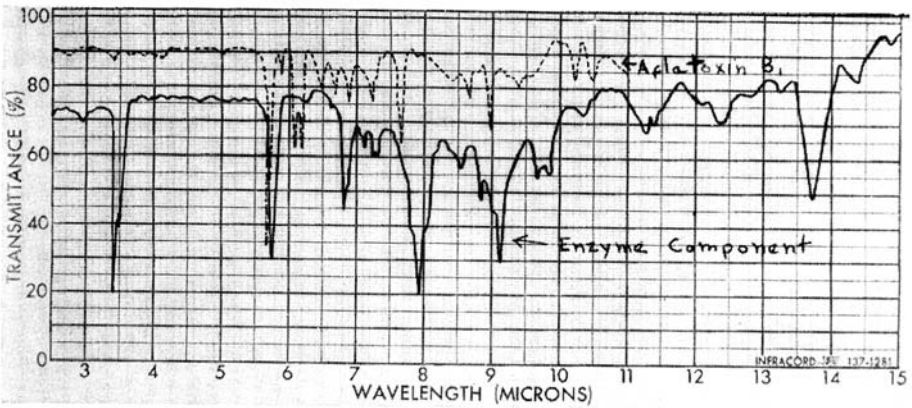


Fig. 5. Infrared spectra of component from chloroform extract of enzyme and of aflatoxin.

the time of assay, should substantially increase the sensitivity, because much more material could be spotted without risk of overloading. However, with the solvents tested so far everything appears to run with the solvent front.

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