Deterioration of Stored Pea Seed by Aspergillus ruber: Partial Purification and Characterization of a Toxin to Peas

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ABSTRACT

A toxin to peas produced by *Aspergillus ruber* was extracted from infected living peas and from cultures of *A. ruber* grown on autoclaved peas. The toxin was partially purified by treatment of aqueous solutions with chromatography on DEAE-Sephadex and Sephadex G-50. The toxin remained in the aqueous phase after treatment with chloroform and butanol. It had a strong negative with chloroform and butanol. It had a strong negative charge at pH values near neutrality, and an apparent molecular weight of 2,000. It was toxic to all cultivars and species of *Pisum* tested, but did not affect seeds of tomato, lettuce, lima bean, squash, or wheat, embryos of squash or wheat, or lima bean embryonic axes. The most highly purified preparations were active against pea embryonic axes at 0.1 μg/ml. Crude extracts of infected living peas, of cultures of autoclaved peas, and of partially purified preparations of some extracts of living peas also contained another component of larger molecular weight. Crude extracts of autoclaved pea cultures of *A. ruber* also had toxic activity to lima bean seeds and embryonic axes, but not to any other seeds. This toxicity to lima beans was lost during purification of the pea toxin.

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Additional key word: mycotoxin.

Earlier papers (2, 6) presented evidence indicating that a toxin produced by *Aspergillus ruber* (Konig, Spieckermann, & Bremer) Thom & Church was primarily responsible for deterioration of stored pea seeds infected with this fungus. Direct physical invasion of the living portions of the seeds by fungal hyphae seemed to be of little importance (6).

A toxin or mixture of toxins was extracted from infected peas, and some of their properties were described. The toxin(s) appeared to be a highly polar, heat-stable substance(s) (2).

This paper describes a procedure for partial purification of the major toxin to peas and some of its biological and physical properties. A brief summary of a portion of this work has been published (5).

MATERIALS AND METHODS.—Crude preparations of the toxin were obtained from cultures of *A. ruber* (Northern Utilization Research and Development Division Culture 52) grown for 7 to 9 days on autoclaved peas in 20 × 150 mm petri dishes or from infected living peas. Dry infected peas, and infected peas placed under conditions favoring germination for 5 to 7 days, were used in different experiments. Peas lost their ability to germinate normally shortly after infection occurred (4, 6). When infected peas were under conditions favoring germination, the quantity of toxin increased approximately fivefold (2). In all cases, the peas were placed in a Waring Blendor with distilled water (2 ml/pea) and homogenized. The resulting slurries were centrifuged at 15,000 *g*, the precipitates discarded, and the supernatant solutions sterilized by filtration through a Millipore filter (0.45 μm pore size). All

fractions collected during purification were also sterilized by Millipore filtration before bioassay. Extracts from living infected peas were only 2 to 10% as active as extracts from cultures of *A. ruber* on autoclaved peas.

I assayed extracts for toxicity by plating surface-sterilized Alaska pea embryonic axes on a medium containing 2 ml of the solution to be tested and 2 ml of a sucrose-mineral salts agar described earlier (2), and noting the amount of necrosis that occurred on the surfaces of the axes. The sucrose-mineral salts medium was amended by adding 50 μg/ml of chloramphenicol and by omitting the penicillin G-dihydrostreptomycin sulfate mixture. In the bioassay, a necrosis index (2) of 3.5 (i.e., when 60 to 65% of the surface of the axis was necrotic) was designated as 100 units of toxicity, and a standard curve was drawn using the logarithmic relationship between the necrosis index and toxin concentration described earlier (2). This relationship was constant throughout the purification.

To determine whether other seeds were sensitive to toxin preparations, tomato seeds (*Lycopersicon esculentum* Mill. 'Heinz 1350'), lettuce seeds (*Lactuca sativa* L. 'Summer Bibb'), squash seeds and embryos (*Cucurbita pepo* L. 'Acorn'), wheat seeds and embryos (*Triticum aestivum* L. 'Red Bobs'), lima bean seeds and embryonic axes (*Phaseolus lunatus* L. 'Cangreen'), and several pea seeds and embryonic axes (*Pisum sativum* L. 'Alaska', 'Dwarf Gray Sugar', 'Mammoth Melting Sugar', and 'Thomas Laxton'; *P. jomardii* Schrank. P.I. 269762; and *P. elatius* Bieb. P.I. 269760) were used. Whole seeds were surface-sterilized for 1 min in 0.8% NaOCl (lettuce and tomatoes) or 3 min in 1.75% NaOCl (squash, wheat, lima beans, and peas) and plated on 1% water agar containing 0, 1, 10, or 50 μg/ml of the purified toxin. The embryos and embryonic axes were removed after a soaking of the seeds under reduced pressure for 4 to 6 hr. The excised embryos and embryonic axes were disinfected with 0.85% NaOCl for 1 min, rinsed with sterile water, and plated on the sucrose-mineral salts agar containing the same concentrations of purified toxin as that used with whole seeds. All tests were also conducted with crude toxin preparations adjusted to similar levels of toxicity to Alaska pea embryonic axes.

Purification procedures were similar for all extracts. They were first concentrated in a rotary evaporator under reduced pressure at 40 C until the toxic activity began to be lost to the precipitate formed during concentration. The concentrated solutions were then extracted with chloroform and butanol by procedures similar to those used for purification of *Helminthosporium* toxins (10, 11). Concentrated solutions were shaken thoroughly with two volumes of chloroform, the resulting emulsion was centrifuged at 25,000 *g*, and the aqueous phase was retained. This procedure was repeated until only traces of denatured protein formed at the chloroform-water interface. The solutions were then extracted with water-saturated 1-butanol and centrifuged at 25,000 *g*. A precipitate usually formed, and more material collected at the interface between the butanol and aqueous phases. Butanol extraction was repeated until no more material precipitated or formed at the interface. The butanol layer, which contained no toxic activity, was discarded.

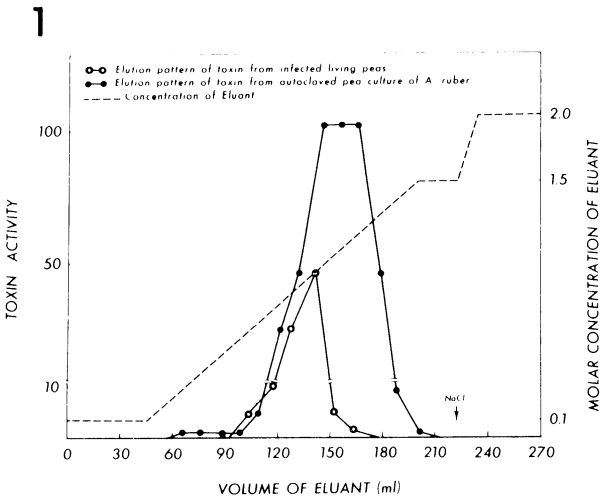
After extraction with chloroform and butanol, the aqueous solutions were applied to DEAE-Sephadex A-25 columns equilibrated with 0.1 M NH_4HCO_3. The column beds were 1.5 × 23 to 24 cm. They were eluted first with 0.1 M NH_4HCO_3 to remove butanol and other materials not bound to the DEAE-Sephadex, then with a linear gradient of 0.1 to 1.5 M NH_4HCO_3, and finally with 2 M NaCl. NaCl was used for the 2-M elution step because NH_4HCO_3 decomposes rapidly in aqueous solutions at concentrations above 1.5 M. Aliquots of the fractions from the DEAE columns were diluted 1:50 or 1:100 to avoid toxicity from butanol, NH_4HCO_3, or NaCl, and bioassayed. The fractions comprising the peak activity were pooled, and either concentrated to approximately 10 ml in a rotary evaporator at 40 C or lyophilized to dryness. The lyophilizate was dissolved in 10 ml of distilled water. Nine ml of the concentrated active fractions were applied to a Sephadex G-50 (50- to 150-μm bead size) column with a bed of 2.5 × 42 cm and eluted with distilled water. Aliquots of the fractions obtained were bioassayed, and the remainder of each fraction lyophilized. The material from the most active fractions was pooled and stored over P_2O_5 at 10-15 C. The material so purified from the *A. ruber* cultures was the partially purified toxin used in the remainder of the experiments. Only toxin from autoclaved pea cultures was used for experiments with purified toxin, since it had the highest activity.

All experiments reported in this paper were replicated at least once with similar results, and most experiments were done three or more times.

RESULTS.—The crude extracts from ungerminated or germinated infected peas or from cultures of *A. ruber* on autoclaved peas were cloudy brown solutions when first prepared, but became clear tan or golden after extraction with butanol and chloroform. No toxic activity was extracted into the chloroform or butanol phases, and about 90% of the original activity was recovered in the aqueous phase after the extractions.

The activity of all extracts was eluted from DEAE-Sephadex columns as similar peaks with 0.9 to 1.2 M NH_4HCO_3 (Fig. 1). When the peak fractions were pooled and concentrated using a rotary evaporator at 40 C, only about 10-20% of the activity was recovered. Concentration by lyophilization, however, gave almost complete recovery of the activity of the peak fractions. Otherwise, samples concentrated by either method were similar.

Toxic activity from crude preparations of autoclaved pea cultures was eluted from Sephadex G-50 columns as a broad plateau with 80 to 140 ml of eluant. After extraction with chloroform and butanol, and DEAE-Sephadex chromatography, all the activity was resolved into a single peak (Peak 2 in Fig. 2) eluted with 130 to 150 ml of eluant. Toxic



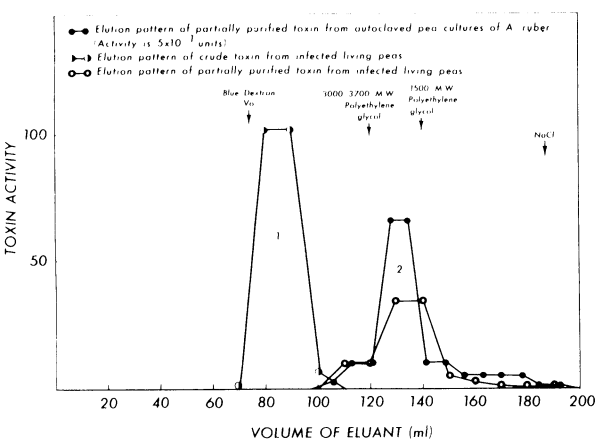


Fig. 1-2. 1) Toxin produced by *Aspergillus ruber*-infected pea seed. Elution patterns of toxins from several sources from DEAE-Sephadex columns. Elution was with NH_4HCO_3 for the first 230 ml, then with NaCl. **2)** Elution patterns of toxins from several sources from a Sephadex G-50 column at different stages of purification. The crude and partially purified toxins from living infected peas are from the same original batch of toxin. The arrows represent the peaks of elution patterns of substances of various molecular weight. In all cases, 9-ml samples were applied to the column.

activity from crude preparations of infected living peas, both germinated and ungerminated, eluted in a single peak (Peak 1 in Fig. 2), with 80 to 100 ml of eluant. After partial purification, activity appeared only as peak 2 in the case of extracts of germinated infected peas; but as two peaks, 1 and 2, in the case of extracts of ungerminated infected peas. Peak 2 had a molecular weight of approximately 2,000 when compared to the elution of different molecular weights of polyethylene glycol.

In all cases, peak fractions from the Sephadex G-50 columns after extraction with chloroform and butanol and chromatography on DEAE-Sephadex were colorless, and when lyophilized were fluffy white substances. Partially purified toxins from different autoclaved pea cultures were active at concentrations of 0.1 to 1 μg/ml. The toxin was not completely purified; the dry weight of the lyophilized fractions from Sephadex G-50 columns was not correlated with elution of activity. The purified preparations from infected living peas were less active.

Since the partially purified toxin from autoclaved pea cultures of *A. ruber* was active at low concentrations and was similar to that from infected living peas, it was used for determination of the biological properties of the toxin.

Crude toxin preparations were active against lima beans and *Pisum* spp., but the purified toxin was active only against *Pisum* spp. Concentrations (up to 50 μg/ml) of the purified toxin had little or no effect

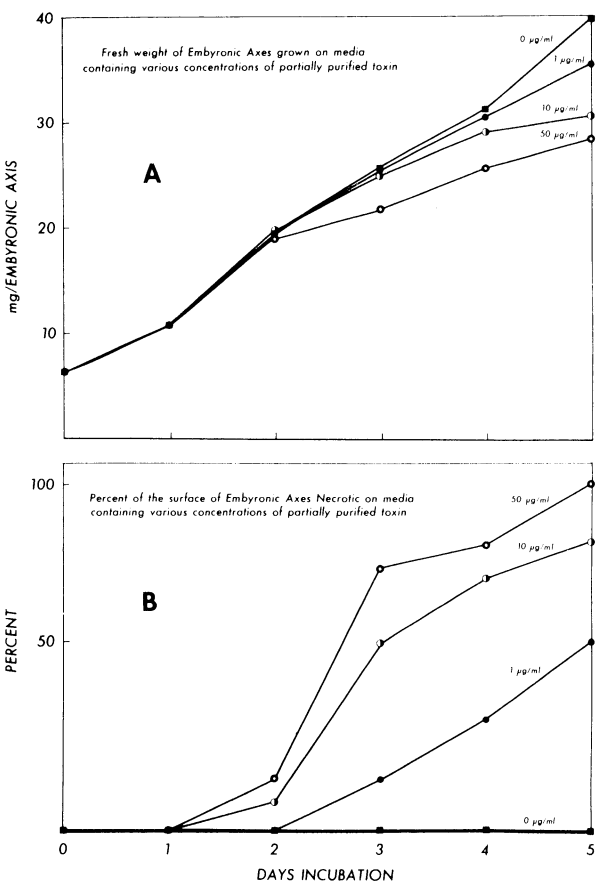


Fig. 3. A) Daily increase in weight of Alaska pea embryonic axes on the sucrose-mineral salts medium containing various concentrations of partially purified toxin. **B)** Percentage of the surface of the Alaska pea embryonic axes that became necrotic after incubation for various lengths of time on the sucrose-mineral salts medium containing various concentrations of toxin.

on lima beans. No toxicity to squash, wheat, lettuce, or tomatoes was found. With all cultivars and species of *Pisum* tested, embryonic axes became very necrotic, and their growth was increasingly inhibited by increased concentrations of toxin (Fig. 3). Whole pea seed germinated normally on water agar containing toxin, but when the tips of the radicles came in contact with the agar, they became necrotic. The radicles remained white and healthy when no toxin was present. Lima bean embryonic axes and whole seeds plated on media containing crude extracts behaved similarly, but were not affected by media containing partially purified toxin.

DISCUSSION.—The toxins extracted from autoclaved pea cultures or from infected living peas appeared to be very similar. They were not denatured, extracted, or precipitated by chloroform or butanol. Each eluted as a similar peak from DEAE-Sephadex, indicating a strong negative charge at pH values near neutrality. When first isolated, the toxins from all sources appeared loosely bound to other materials as indicated by the change from larger to smaller molecular weight as purification progressed. Apparently, the toxin extracted from ungerminated peas was not completely dissociated from the material to which it was bound, since two peaks of activity were detected when the purified toxin was chromatographed on Sephadex G-50. It is possible, however, that two toxins are present in the extracts from ungerminated peas. If so, their single peaks when eluted from DEAE-Sephadex and their solubility properties (cf.2) suggest that the two toxins are similar except for the difference in molecular weight. Complexes between molecules of biological interest are common and may present difficulties in analyzing systems in which they occur (3, 7, 9).

The toxin acted slowly on sensitive tissues. In pea embryonic axes, the initially observed effect was necrosis of the outer surface of the axes; a decrease in the rates of growth was not seen until necrosis of the surface of the axes was severe (Fig. 3). The necrosis of the surface appeared to be a hypersensitive reaction, and the outer cells of the axes became filled with a granular brown material. This reaction may have slowed uptake of the toxin and may have delayed death of the whole axes.

The specificity of the purified toxin was surprising. Members of the *Aspergillus glaucus* group, which includes *A. ruber*, are involved in deterioration of a large variety of seeds (1). A toxin probably has a primary role in deterioration of stored pea seeds infected with *A. ruber* (2, 6). Only the toxin described in this paper has been found in peas infected by *A. ruber*. If toxins induce deterioration of other stored seeds, they should be found in other seeds infected by storage fungi. In this work, we found a toxin injurious to lima beans in crude extracts but not in purified preparations, suggesting that *A. ruber* may be able to produce other relatively specific toxins. Moreno-Martinez & Christensen (8) showed that in certain lines of corn (*Zea mays* L.), a high percentage of the kernels may be infected with storage fungi with little loss in percentage germination. One explanation for these observations is that corn lines apparently resistant to deterioration are resistant to the toxins produced by *A. glaucus* or *A. restrictus*. If toxins produced by storage fungi are the primary determinants of seed deterioration, it may be possible to develop rapid bioassay systems for evaluating resistance of various cultivars of important seed crops for resistance to seed deterioration by storage fungi.

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