Soybean Lipoxygenase Is Active on Nonaqueous Media at Low Moisture: A Constraint to Xerophilic Fungi and Aflatoxins?

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ABSTRACT: Previous workers have reported that certain products of the lipoxygenase pathway are detrimental either to the development and growth of *Aspergillus* species or to aflatoxin production by these organisms. Since *Aspergillus* often thrives on "dry" stored grains, depending on the level of the relative humidity, we sought to determine if lipoxygenase could catalyze the oxidation of linoleic acid on these "dry" substrates equilibrated at various relative humidities. A desiccated model system, previously adjusted to pH 7.5, was composed of soybean extract, linoleic acid, and cellulose carrier. The model system was incubated for up to 24 h at four relative humidities ranging between 52 and 95% to determine the extent of oxidation catalyzed by lipoxygenase, compared with heat-inactivated controls. Oxidation in the active samples was much greater than in the controls at all relative humidities, and oxidation was principally enzymatic as demonstrated by chiral analysis of the linoleate hydroperoxides formed. The main product was 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, accompanied by a significant percentage of 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid. Since the products became more racemic with time of incubation, autoxidation appeared to be initiated by the lipoxygenase reaction in dry media. Additionally, the biological relevance of lipoxygenase activity was tested under these xerophilic conditions. Thus, enzyme-active and heat-inactivated defatted soy flour amended either with or without 3.5% by weight linoleic acid was inoculated with fungal spores and incubated at 95% relative humidity. Although fungal growth occurred on all treatments, samples inoculated with *Aspergillus parasiticus* showed significantly less aflatoxin in the enzyme-active samples, compared to inactivated flour. Addition of linoleic acid had little effect, possibly because the defatted soy flour was found to contain 1.7% residual linoleic acid as glyceride lipid.
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KEY WORDS: Aflatoxin, *Aspergillus,* hydroperoxylinoleic acid, lipoxygenase, low moisture, soybean.

Because aldehydes generated by the lipoxygenase pathway are inhibitory to fungal growth (e.g., Ref. 1), it has been hypothesized that oilseed-feeding fungi trigger a cascade to aldehydes by producing lipase to utilize seed triacylgylcerol. Lipase production by oilseed-contaminating fungi, including *Aspergillus flavus*, is well known (2,3). When the released polyunsaturated fatty acids are oxidized by plant lipoxygenase into fatty acid hydroperoxides, it is theorized that the hydroperoxides are converted into various metabolites, including aldehydes formed by catalysis with hydroperoxide lyase (4–6). Aldehydes inhibit growth, aflatoxin production (6–9), and spore germination (5) of *A. flavus*. After the pioneering work of Major *et al*. (1), many investigators documented the inhibitory effect of aldehydes on the growth of various fungal species. Goodrich-Tanrikulu *et al*. (10) showed that methyl jasmonate, another lipoxygenase pathway metabolite, also inhibits aflatoxin production by *A. flavus*.

Soybean is known to be resistant to aflatoxin accumulation, with reported low incidence from numerous samples collected from different locations (11,12). That soybeans are an exceptionally rich source of lipoxygenase leads some investigators to believe the enzyme is a deterrent to the incidence of aflatoxin. However, soybeans can serve as a good substrate for *Aspergillus* growth and aflatoxin formation under conditions of high moisture (13) or mechanical disruption (14,15).

More recently, Burow *et al*. (16) showed that one soybean lipoxygenase product, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, inhibited growth and aflatoxin production in *Aspergillus* species at micromolar concentrations, while another product of certain other lipoxygenases, 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid, did not inhibit growth or aflatoxin production. They showed that 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid reduced aflatoxin production through the suppression of aflatoxin biosynthetic gene transcription.

If lipoxygenase has a reasonable chance to be physiologically important, then it must be capable of catalyzing oxidation in the xerophilic conditions under which storage fungi can grow. Although there are reports that lipoxygenase can be active in "dry" media (17–19), none of these studies examined the chirality of products, a necessary requirement to prove enzyme action. Inasmuch as Brockmann and Acker (18) found equal quantities of 9- and 13-hydroperoxides in

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the lipoxygenase-catalyzed oxidation of linoleic acid at 65% relative humidity, this reaction could have been due to autoxidation which typically gives a 1:1 mixture of 9- and 13-hydroperoxides.

The current study found a definite stereospecific oxidation of linoleic acid by soybean-amended cellulose incubated at various relative humidities, compared with heat-inactivated soybean controls. A very small chiral preference was even obtained after heat-inactivation, indicating either an endogenous activity prior to heat inactivation or incomplete inactivation. Further, we found that the fatty acid hydroperoxides formed were predominantly the 13*S*-stereoisomer, providing proof that a significant proportion of their origin had to be enzymic. Possible biological relevance to *Aspergillus* and aflatoxin in soybean was assessed.

EXPERIMENTAL PROCEDURES

Lipoxygenase reaction conditions. The "dry" medium used in this study was a matrix of soybean extract, containing lipoxygenase activity, vacuum-dried on cellulose [20 μ cellulose (Sigmacell, type 20™) from Sigma, St. Louis, MO]; this soybean extract on cellulose was mixed with an equal weight of cellulose containing linoleic acid, 99+% pure (Nu-Chek-Prep, Elysian, MN). The enzymic oxidation of linoleic acid in the matrix at 20°C and various relative humidities was measured over time. A range of relative humidities (20) was obtained by suspending the samples over saturated solutions of the following salts (% relative humidity): $NaHSO_4$ (52%); $NaClO_3$ (75%); KBr (84%); Na_2HPO_4 (95%). Since the bulk of the dried matrix was cellulose (~90%), the time required for equilibrium of moisture adsorption by cellulose was determined. Cellulose dried at 110°C for 1 h was weighed, placed in the relative humidity chambers at 20°C, and withdrawn for reweighing at various times.

The source of lipoxygenase activity in this study was Williams (cv.) 1993 defatted soybeans (crop of 1993 stored at 4°C). Soybeans (10 g) were ground in a coffee grinder and then immersed in 200 mL hexane for 1 h on dry ice. The hexane-defatted meal was collected by vacuum filtration, washed with hexane, and air-dried. The defatted meal was further crushed by a mortar and pestle, and extracted again in 200 mL hexane for 1 h over dry ice, filtered, and washed with hexane and air-dried. All experiments were done with freshly prepared defatted soybeans.

Aqueous extracts were prepared by soaking 4 g of the defatted soybean meal 10 min in 40 mL deionized water on ice, and then homogenizing by a Polytron Homogenizer (Kinematica AG Littau, Switzerland) for 0.5 min on ice. The homogenate was adjusted to pH 7.5 with 1 N KOH and centrifuged 15 min at $10{,}000 \times g$. A small amount of fatty material floating on top of the centrifugate was removed by aspiration. The supernatant obtained in this way (about 25 mg protein/mL) was added to cellulose in the amount of 1 mL/g cellulose. This mixture (10 g total) was placed on a 9-cm diameter petri dish in a vacuum desiccator over Drierite desiccant and dried in the dark for 18 h using house vacuum (0.25 ± 0.1 atm). It was determined that complete drying occurred in less than 9 h under these conditions.

Linoleic acid [containing 0.052% butylated hydroxytolulene (BHT) to inhibit autoxidation] was also incorporated into a cellulose matrix. An aqueous suspension of BHT-amended linoleic acid was prepared by suspension in water followed by sonication and adjustment to pH 7.5. This solution had to be sonicated for a sufficient time to obtain a uniformly cloudy suspension (pH 7.5 is approximately the pK_a of linoleic acid). The linoleate solution (5 mL at 50 mg linoleic acid/mL) was then mixed with 5 g cellulose and dried in the vacuum desiccator in the dark for 18 h as described above for the soybean/cellulose preparation.

Lipoxygenase activity commenced after an equal weight of dried soybean extract–cellulose and dried linoleic acid–cellulose were mixed by a mortar and pestle. The mixture was incubated in open petri dishes in the humidity chambers at 20°C in the dark. Aliquot samples (0.2 g) were removed for analyses at 0 time and after 1, 3, 7, and 24 h. The samples were immediately added to 0.5 mL diethyl ether containing 5 mg triphenylphosphine to reduce hydroperoxides to their corresponding hydroxides and stored in the freezer for subsequent analyses. Control reactions followed the procedure described above, except the soybean extract was heated for 15 min at 90°C to inactivate lipoxygenase.

Analysis of product hydroperoxide fatty acids. For analysis of the oxidized linoleic acid in the diethyl ether–cellulose mixture, 10 μL acetic acid and 0.15 mL methanol were added to convert the carboxylate to carboxylic acids, and this mixture was filtered through glass wool to remove the cellulose. The filtrate solution containing fatty acids was treated with diazomethane to form methyl esters. The esterified fatty acids were chromatographed on precoated silica gel 60 F 254 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany), 20×20 cm $\times$ 250 μm using hexane/diethyl ether (3:2, vol/vol) as the developing solvent. The ultraviolet (UV)-absorbing zone corresponding to a methyl 13-hydroxyoctadecadienoate standard was scraped taking care to include an area of the plate at a lower R_f where methyl 9-hydroxyoctadecadienoate *E,E*-diene isomer was expected to migrate. The scrapings were extracted with ethyl acetate, and extracts were dried under nitrogen and redissolved in 3 mL hexane. Hexane solutions were used for high-performance liquid chromatography (HPLC), to determine isomeric composition, and for gas chromatography (GC), to quantitate the methyl hydroxyoctadecadienoates.

Straight phase-HPLC (SP-HPLC) was used to separate methyl hydroxyoctadecadienoate isomers in the following elution order: methyl 13-hydroxy-9*Z*,11*E*-octadecadienoate, methyl 13-hydroxy-9*E*,11*E*-octadecadienoate, methyl 9-hydroxy-10*E*,12*Z*-octadecadienoate, and methyl 9-hydroxy-10*E*,12*E*-octadecadienoate as previously reported (21). SP-HPLC was completed using a Rainin Instrument Co. (Woburn, MA) Microsorb column (5 μ silica, 4.6×250 mm) with hexane/2-propanol (99:0.85 vol/vol) elution at 0.75

mL/min. The separated peaks were collected, and the two *E,Z*-diene isomers were separated into *R* and *S* isomers by chiral phase-HPLC (CP-HPLC). CP-HPLC was completed with a Chiracel OB column from J.T. Baker, Inc. (Phillipsburg, NJ; 4.6 × 250 mm, 10 μm particle size) with hexane/2-propanol (97:3, vol/vol) elution at 0.6 mL/min as previously reported (22). Peaks were detected and the relative percentage of each isomer was determined by an UV spectrometer set at 232 nm.

GC was utilized to determine the total amount of methyl hydroxyoctadecadienoates in each sample by analyzing their trimethylsilyloxy (OTMS) ethers in the presence of a tetracosane (Aldrich Chemical Co., Milwaukee, WI) internal standard. GC was completed with a Hewlett-Packard Model 5890 gas chromatograph equipped with flame-ionization detection and a SPB-1 (dimethylpolysiloxane phase) capillary column (30 m × 0.32 mm; film thickness, 0.25 μm) from Supelco (Bellefonte, PA). Temperature programming was from 160 to 260°C at 5°C/min at a He flow of 2 mL/min. Injector and detector temperatures were 270 and 280°C, respectively. OTMS ethers were synthesized by addition of chlorotrimethylsilane/hexamethyldisiloxane/pyridine (3:2:2, vol/vol/vol). After 10 min reaction, the reagent was evaporated with a stream of N_2, and hexane was added for GC analysis.

Fungal growth methods. Aspergillus parasiticus ATCC 56775 (American Type Culture Collection, Rockville, MD) was maintained as a silica (RT) stock. Inoculum was prepared by growing the fungus at 30°C on potato dextrose agar. Conidia were harvested with an aqueous solution of 0.1% (vol/vol) Tween 20 and adjusted to the desired inoculum concentration (10^8 conidia/mL).

To serve as a growth medium, active and heat-inactivated defatted soybean flours were utilized with or without 3.5% by weight of added linoleic acid. Soybeans (cv. Williams) were ground by a coffee mill and then extracted with hexane; the grinding/extraction process was repeated until the hexane extract was colorless (5 to 7 times). Water was added to the defatted flour, adjusted to pH 7.5 with 1 M KOH, and then lyophilized to serve as the enzyme "active" sample. Another portion of flour was adjusted to pH 7.5, and then boiled for 20 min to "heat-inactivate" the flour before lyophilization. Extraction efficiency of hexane was examined by immersion of 2 g soy flour in 50 mL $CHCl_3/CH_3OH$ (2:1, vol/vol) for 2 d at –20°C with occasional stirring, followed by recovery of the organic solvent by filtration through sintered glass, and washing of the residual flour with $CHCl_3/CH_3OH$ (2:1, vol/vol). The combined extract was washed with water equivalent to one-third the volume of solvent. Extracted lipid was assessed by two-dimensional TLC with $CHCl_3/CH_3OH/H_2O$ (65:25:4, vol/vol/vol) followed by hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol). The lipid was then saponified by heating with 2 mL 0.5 N NaOH in methanol followed by the addition of 2.5 mL 14% BF_3 in CH_3OH for 5 min to prepare fatty acid methyl esters, which were recovered by hexane extraction by adding hexane and water to the reaction mixture.

Soy flour was defatted to inhibit lipoxygenase activity from acting on endogenous polyunsaturated fatty acids before the addition of *Aspergillus* inoculum. Only at the time of inoculation was linoleic acid added back at 3.5% by weight to the soy flour, on the low side of what would normally be present as triglyceride. That is, soybean is normally 20% oil with linoleic acid being 51–52% of the fatty acid composition of the oil (23).

Fungal cultures were incubated in a 10-L enclosed glass incubator adjusted to 95% relative humidity with a solution of saturated Na_2HPO_4. Twelve sterile 9-cm diameter glass petri plates containing four sterile 3.1-cm diameter caps were placed in the incubator. Saturated Na_2HPO_4 (2 mL) was placed in one cap for each glass petri plate. The other three caps contained the soybean/fungal mixtures described as follows.

Aspergillus parasiticus was grown in (i) active soybean flour, (ii) active soybean flour plus 3.5% linoleic acid by weight, (iii) deactivated soybean flour, or (iv) deactivated soybean flour plus 3.5% linoleic acid. For each treatment, 225 mg of soy flour was mixed with 7.9 mg of linoleic acid (from Sigma Chemical Company) under liquid nitrogen using a mortar and pestle. Linoleic acid was dissolved in 1 mL methanol prior to mixing with soy flour to allow for more even mixing. The soy flour treatments without linoleic acid were mixed with 1 mL methanol as a control. In all treatments, methanol was allowed to evaporate through lyophilization prior to inoculation with *A. parasiticus*.

After lyophilization, the soy flour mixtures were placed in the 3.1-cm caps (225 mg soy flour/cap), three caps of one treatment per glass petri dish. Each treatment consisted of three petri dishes for a total of nine caps/treatment. Sterile 1.3-cm diameter filter discs were immersed in an *A. parasiticus* spore suspension adjusted to cover the disc with 10^6 spores/disc. Discs were air-dried under a biosafety hood for ~1 h to evaporate water and then one disc was placed in each cap. The incubator containing the glass petri dishes was set at 28°C, and one cap from each glass petri dish was removed and assayed for spore production and aflatoxin content after 2, 3, and 4 d of incubation. All treatments were triplicated.

For extraction and quantification of conidia and aflatoxin, each cap was flooded with 10 mL of 0.1% Tween water in a small beaker, and the contents (soy flour and fungus) were dislodged, transferred to 15-mL test tubes, and vortexed for 1 min to free all conidia. Conidia were then counted using a hemacytometer. For aflatoxin analysis, 100 μL of the supernatant was mixed with 100 μL 70% methanol following Campbell and White (24).

Enzyme-linked immunosorbent assay (ELISA) detection of aflatoxin B_1 (AFB_1). The common practice of measuring AFB_1 rather than all four aflatoxins (AFB_1, AFG_1, AFB_2, AFG_2) was followed for this study. AFB_1 was analyzed from the filtrate by indirect competitive ELISA as described previously (25). Aflatoxin ELISA reagents were obtained from Sigma Chemical Company.

Statistical analysis. Each fungal treatment was represented by three replicates, and values presented in this paper were means of these replicates. ELISA quantifications of AFB_1 and

spore counts were analyzed by two-way analysis of variance. Differences between means were evaluated by Fisher's protected least squares difference.

RESULTS AND DISCUSSION

Lipoxygenase activity at low moistures. Because lipase activity is widely known to occur under conditions of low moisture (e.g., Ref. 26), it is likely that the first step of the cascade can take place; that is, the hydrolysis of triacylglycerol. Although many seed lipases are quiescent, it is well-known that oilseed parasitic fungi produce lipase capable of acting at low moisture levels (2).

The next step of the cascade is lipoxygenase oxidation of polyunsaturated fatty acids, but it has not been conclusively shown that lipoxygenase can react in low-moisture seed meals. If lipoxygenase plays a role in the defense against fungal growth, then the enzyme should function at the xerophilic conditions under which certain fungi, like *Aspergillus*, grow. The question of lipoxygenase activity at low moisture content was answered by this study. Incubations of dried soybean extract plus linoleic acid incorporated on a cellulose matrix at relative humidities ranging from 52 to 95% all showed hydroperoxide-forming activity that greatly exceeded the heat-inactivated soybean control. In early studies (data not shown) it was found that incorporation of BHT was desirable to suppress a background of linoleic acid autoxidation, thereby better revealing the oxidation due to the enzymic component. Figure 1 shows that oxidation increased with time with little dependence on relative humidity, except that oxidation at 95% relative humidity was greater. The heat-inactivated controls showed little oxidation with time. In these experiments the dried samples (overnight vacuum-dried over desiccant) were placed immediately in humidity chambers before the samples had had time to reach moisture equilibrium. This initial lack of equilibrium conditions may have accounted for the relatively large coefficient of variation obtained in these experiments (Fig. 1).

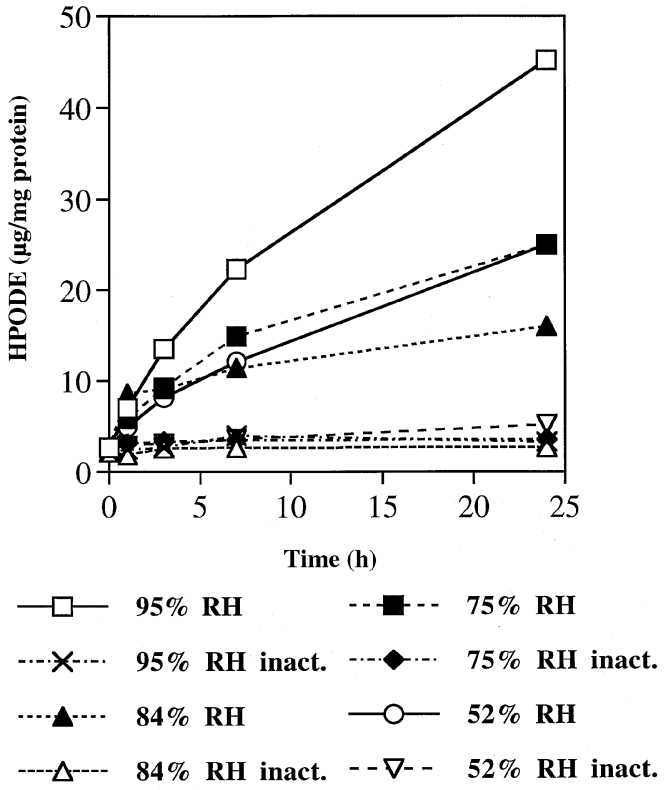

FIG. 1. Formation of 9- and 13-hydroperoxyoctadecadienoic acids (HPODE) from oxidation of linoleic acid on cellulose media incorporated with either dried soybean extract or dried heat-inactivated soybean extract. After drying the soybean extract–cellulose and linoleate–cellulose overnight, a mixture of the two was immediately placed in RH chambers (time zero), and timed measurements were taken thereafter. The amount of HPODE was analyzed by gas chromatography after triphenylphosphine reduction to their corresponding hydroxyoctadecadienoic acids followed by methyl esterification and formation of trimethylsilyloxy ethers; values are means of triplicate determinations with a mean coefficient of variation of 57%. Abbreviations: RH, relative humidity; inact., heat-inactivated.

Detailed analysis of the triphenylphosphine-reduced hydroperoxides by SP-HPLC and CP-HPLC showed that there was preferential formation of certain stereoisomers, showing the involvement of enzymic activity. As seen in Table 1, the principal isomer was methyl 13*S*-hydroxy-9*Z*,11*E*-octadecadienoate followed by methyl 9*S*-hydroxy-10*E*,12*Z*-octadecadienoate, as expected for oxidation by a mixture of soybean lipoxygenase isozymes at pH 7.5 (27). As a comparison, linoleic acid in the absence of soybean extract showed a completely racemic mixture of stereoisomers of the *E,Z*-dienes (Table 1). Because the *E,E*-diene isomers arise from peroxyl radicals involving a carbon-oxygen bond scission, it was surmised that these isomers were racemic; thus, only a few samples were analyzed by CP-HPLC to confirm that they were racemic *R,S* mixtures. A short-time (15 min) oxidation by an aqueous soybean extract at pH 7.5 afforded more isomeric purity than the low-moisture treatments (Table 1), indicating that the "dry" reaction had an autoxidation component probably initiated by enzymically formed hydroperoxides. As a function of time, there was an overall decline in stereochemical selectivity, as well as a trend toward increased *E,E*-diene formation. Both of these observations indicate an autoxidation component. As mentioned above, it is known that *E,E*-diene hydroperoxides are a consequence of peroxyl radicals formed either kinetically through autoxidation (28) or from peroxyl radical reactions of pre-formed hydroperoxides (29).

An experiment was completed with oven-dried cellulose placed in humidity chambers to determine the time required for moisture equilibrium. Approximately 7 to 24 h was required for the cellulose sample to reach equilibrium at the various relative humidities (data not shown). Thus, as expected, a 24 h preequilibration of the linoleic acid/cellulose and soybean/cellulose samples incubated separately at the various humidities increased both the rate and final amount of hydroperoxides after admixture (Fig. 2). Also, the coefficient of variation obtained for these data was much reduced compared to the nonequilibrated experiments shown in Figure 1. After preequilibration there was a trend of increased

TABLE 1
Oxidation Specificity of Linoleic Acid Oxidation by Dried Soybean Extracts on Dry Cellulose Media Incubated at Various Relative Humidities Compared with Linoleic Acid Oxidation by an Aqueous Soybean-Linoleic Acid Reaction[a]

			Regio- and stereochemical composition (%)					
			13-Hydroperoxides			9-Hydroperoxides		
Enzyme source	Incubation time (h)	Relative humidity (%)	*S,Z,E*	*R,Z,E*	*RS,E,E*	*S,Z,E*	*R,Z,E*	*RS,E,E*
Linoleic acid/cellulose[b]	24	95	17.8	17.6	15.6	17.3	17.2	14.6
Dried soybean extract	0[c]	N.A.	45.0	12.9	8.6	16.4	10.0	7.0
	1	52	44.6	13.8	7.6	18.7	10.3	4.9
	3	52	38.0	15.1	8.8	27.6	7.2	9.3
	7	52	30.2	19.4	7.3	26.1	10.0	7.0
	24	52	30.2	18.6	10.4	18.0	17.2	8.1
	1	75	39.6	14.7	6.6	22.2	10.2	6.7
	3	75	33.2	15.5	7.5	26.3	9.6	7.9
	7	75	33.5	15.4	8.5	24.3	9.4	8.9
	24	75	29.0	16.7	9.5	21.8	12.9	10.2
	1	84	38.7	14.4	8.7	19.0	11.3	7.8
	3	84	41.4	10.4	6.6	24.7	10.5	6.3
	7	84	29.8	20.7	8.4	24.9	7.6	8.4
	24	84	29.4	15.9	11.5	22.5	9.3	11.7
	1	95	53.4	14.7	5.6	14.0	7.7	4.2
	7	95	36.1	13.2	10.7	17.9	11.1	11.0
	24	95	38.8	15.3	11.8	16.4	12.3	10.0
Heat-inactivated and dried soybean extract[d]	27	95	19.6	17.0	14.2	19.7	16.7	12.8
Soybean extract[e]	0.25	Aqueous	59.1	6.2	4.8	15.9	7.8	6.2
Heat-inactivated soybean extract[e]	0.25	Aqueous	45.0	9.4	9.5	20.5	7.3	8.3

[a]The "dry" reactions were not preequilibrated before incubating at the relative humidities used, and samples were taken at various times for analysis. All reactions were adjusted to pH 7.5.
[b]Linoleic acid/cellulose only without butylated hydroxytoluene (BHT). BHT was omitted to promote autoxidation.
[c]Sampled immediately after mixing linoleate/cellulose and dried soybean extract/cellulose (time zero).
[d]Soybean extract was heated to 100°C for 15 min prior to drying on cellulose. Amount of 9- and 13-hydroperoxyoctadecanoic acid (HPODE) analyzed was 2.28 μg/mg protein which was comparable to the value obtained with heat-inactivated soybean incubated for 24 h at 95% relative humidity (Fig. 1).
[e]Soybean extract (0.2 mL) (or heat-inactivated soybean extract; see footnote *d* for heat treatment) was incubated with 5 mg linoleic acid (1 mM) in 17.8 mL 50 mM HEPES buffer, pH 7.5, at 25°C. Total HPODE in the reaction catalyzed by heat-inactivated soybean extract was low (1.49 μg/mg protein), compared to a relatively large quantity of HPODE (52.7 μg/mg protein) catalyzed by unheated soybean extract.

oxidation with increased relative humidities. The exception was a burst of activity in the 52% relative humidity sample after 7 h. This possibly can be explained by accelerated autoxidation often seen at low humidities (30). It is theorized that low humidities concentrate metal contaminants that initiate autoxidation. However, intermediate moistures can promote a reversal to increased autoxidation; that is, Labuza *et al*. (31) found greater oxidation at 75 than 52% relative humidity, a result not apparent in this study. Table 1 shows that the product mixture obtained at relative humidity of 52% was more racemic than that obtained at other humidities indicating greater participation of autoxidation.

Biological relevance. To test the biological relevance of lipoxygenase activity at 95% relative humidity, we inoculated defatted soybean flour ±3.5% linoleic acid with fungal spores. Preliminary experiments with the lipoxygenase test system amended with cellulose provided insufficient nutrients to support abundant fungal growth (data not shown); therefore, soybean flour provided adequate media. For this purpose, defatted soybean flour was suspended in water and adjusted to pH 7.5. One sample was heat-inactivated by boiling before drying, and the other was lyophilized directly. The dried flour samples were reconstituted either with or without 3.5% by weight linoleic acid. Initially, the treatments were incubated several days at 95% relative humidity at room temperature exposed to spores from a mixed fungal culture. Extensive fungal growth developed on all treatments, and visual examination usually showed little difference between the heat-inactivated and enzyme-active samples (data not shown). In regard to general fungal growth, other defenses, such as the seed coat and cellular integrity, may provide more important means of protection under these conditions. In fact, we found that intact soybean seeds were resistant to fungal growth under the same conditions under which lush fungal growth occurred on the linoleic acid-amended and unamended soy flour samples. Previous work by Stössel (15) showed that soybeans with damaged seed coats supported vigorous *Aspergillus* growth while beans with intact seed coats did not. Although Stössel found that untreated soybeans with intact seed coats were the most resistant to fungal attack, even autoclaved beans with intact seed coats were almost as resistant (15).

However, the preliminary experiments above did not an-

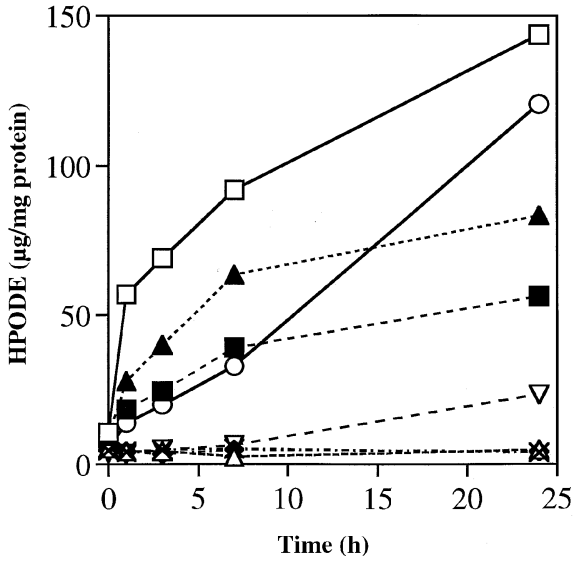

FIG. 2. Formation of HPODE from oxidation of linoleic acid on cellulose media incorporated with dried soybean extract or dried heat-inactivated soybean extract after preequilibration at various RH. After drying the soybean extract-cellulose and linoleate-cellulose overnight, each was separately incubated 24 h at various RH. These preequilibrated soybean extract–cellulose and linoleate-cellulose powders were then mixed and immediately placed in RH chambers (time zero), and timed measurements were taken thereafter. The amount of HPODE was analyzed by gas chromatography after triphenylphosphine reduction to their corresponding hydroxyoctadecadienoic acids followed by methyl esterification and formation of trimethylsilyloxy ethers; values are means of duplicate determinations with a mean coefficient of variation of 14%. For abbreviations and symbols see Figure 1.

TABLE 2
Effects of Soybean Flour and Linoleic Acid on Spore and Aflatoxin Production in *Aspergillus parasiticus* ATCC 56775

Treatments[a]			
Soybean flour	Linoleic acid (vol/wt)	Spores/mL[b]	Aflatoxin (ng/mg)[c]
Day 2			
Active	3.5 %	1.5×10^6	18[a]
Active		1.5×10^6	6[a]
Deactivated	3.5 %	3.8×10^5	87[b]
Deactivated		2.0×10^5	95[b]
LSD[d]		1.4×10^6	40
Day 3			
Active	3.5 %	$7.2 \times 10^{6a,b}$	20[a]
Active		$4.4 \times 10^{6b,c}$	46[b]
Deactivated	3.5 %	2.9×10^{6c}	176[c]
Deactivated		2.7×10^{6c}	180[c]
LSD		3.4×10^6	25
Day 4			
Active	3.5 %	1.5×10^7	48[a]
Active		1.2×10^7	35[a]
Deactivated	3.5 %	7.9×10^6	183[b]
Deactivated		7.3×10^6	189[b]
LSD		1.0×10^7	61

[a]Activated = soyflour was not heated and lipoxygenase activity should be present; deactivated = soyflour was heated and lipoxygenase activity was absent.
[b]Conidia were counted in a 10-mL extract of each treatment. Values presented are means of three replicates.
[c]ng of aflatoxin/mg of soybean flour. Values presented are means of three replicates.
[d]Means of triplicates were analyzed using Fisher's protected LSD at $P = 0.05$. Signficant differences between treatments are indicated by different roman superscript letters (a, b, and c).

swer the important question of aflatoxin accumulation under these conditions. In experiments similar to those described above, heat-inactivated and active pH 7.5 soybean flour plus or minus 3.5% linoleic acid was inoculated with *A. parasiticus* spores to determine relative fungal sporulation and AFB_1 production. Heat inactivation of soybean flour had a highly significant effect on *Aspergillus* aflatoxin production for all 3 d and on *Aspergillus* sporulation on day three (Table 2). There was an approximate five- to tenfold increase in aflatoxin production in the heat-inactivated soy flour treatments regardless of the presence or absence of linoleic acid for all 3 d. The addition of linoleic acid showed an additional significant effect on day three where the active soy flour plus linoleic acid treatment contained the least amount of aflatoxin. The sum of the data showed a correlation of increased aflatoxin and decreased sporulation in inactivated vs. activated soy flour treatments.

Previous research showed that the products of plant lipoxygenases, hydroperoxy linoleic acids, have a significant effect on mycotoxin production in *Aspergillus*, including aflatoxin production by *A. parasiticus* (16). In particular, the product of linoleic acid oxidation by soybean lipoxygenase-1, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, resulted in decreased aflatoxin production by *A. parasiticus*. However, in these experiments the fungus was grown in liquid shake cultures, an environment where it is unable to sporulate, so the effects of this compound on fungal development were not recorded. More recently, studies have determined that both 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid and linoleic acid affect *Aspergillus* sporulation on solid growth medium (31). In *A. flavus* and *A. parasiticus*, direct application of 0.1 and 1.0 mg of both compounds stimulates conidial production (A.M. Calvo, L.L. Hinze, H.W. Gardner, and N.P. Keller, unpublished work). Here we have attempted to determine if soybean lipoxygenase activity in soy flour might affect *Aspergillus* aflatoxin and spore production in a simulation of 95% relative humidity storage conditions. Based on the studies mentioned earlier (16,32) we predicted that the active soy flour plus linoleic acid treatments would result in increased sporulation and decreased aflatoxin production.

Such a result was observed for day three but not day two or day four (Table 2). Instead, soy flour heat treatments had a much more significant effect on both sporulation and aflatoxin production than linoleic acid. Enzyme-active soy flour always resulted in less aflatoxin and more spore production. Assigning a precise mechanism leading to this biological result is difficult for several reasons. First, although the flour was defatted, the residual lipid was determined to be 3.9% of the total flour weight, and was composed roughly of equal amounts of phospholipid and triglyceride by TLC analysis.

GC of the fatty acid methyl esters from residual lipid showed that linoleic acid was 61% of the methyl esters and, based on the internal standard, 19:0, linoleic acid was present in the soy flour at 1.7% by weight. After hydrolysis by lipases, this glyceride linoleic acid would serve as a substrate for lipoxygenase, thus obscuring the need for additional linoleic acid. Thus, it is likely that both active treatments would be expected to present results like those in Table 2. Second, heat treatment of soy flour deactivates not only lipoxygenase but also other enzymes in the flour that may have contributed to the results shown in Table 2. We also note that the total amount of linoleic acid added to each treatment (~7.9 mg) was dispersed in 225 mg of soy flour, thus diluting the linoleic acid to 35 mg/g of flour. This was somewhat less than amounts found to stimulate sporulation as described by Calvo and Keller (32) and may have influenced the results. Regardless, the notable reduction of aflatoxin production associated with the active soy flour treatments supports the view that soybean lipoxygenase may be one factor which contributes to the relatively low levels of aflatoxin contamination associated with soybean *in situ*. Interestingly, preliminary studies showed a soybean line containing a nonfunctional lipoxygenase-1 gene to be more susceptible to aflatoxin contamination than a wild-type line (Keller, N.P., unpublished data).

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