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EFFECTS OF LIPOXYGENASE INHIBITORS ON THE FORMATION OF VOLATILE COMPOUNDS IN WHEAT

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; lipoxygenase inhibitors; acetonylacetone *bis*-phenylhydrazone; volatiles.

Abstract—An inhibitor of lipoxygenase, acetonylacetone *bis*-phenylhydrazone (AABPH), markedly reduced the formation of the major volatile C_6 products isolated by reduced pressure steam distillation–hexane extraction of wheat plant homogenates. The compound was active on plant extracts containing lipoxygenase activity and completely inhibited activity at a concentration of 2.5 μM. Two other lipoxygenase inhibitors, phenidone and nordihydroguaiaretic acid, were not as active as AABPH. Linoleate added prior to homogenization increased the quantities of certain lipoxygenase derived volatiles, whereas heat treatment caused a marked reduction in the production of all volatile compounds.

INTRODUCTION

Extensive studies have been conducted on the isolation and chemical identification of volatile compounds produced by plants [1–3]. Although large numbers of compounds have been identified, many questions remain about the synthesis of these compounds and their precursors in plant tissue. Information on the generation of volatiles may provide a basis for altering quantities of biologically active volatiles such as flavour and off-flavour components, and compounds involved in plant host–parasite interactions. Enzyme-specific inhibitors may be used to determine the origin of volatiles.

Recently, a ketone hydrazone was discovered which blocks the action of arachidonic acid lipoxygenase in blood platelets; this enzyme may be involved in the inflammatory response in animal systems [4]. Subsequently, several additional ketone hydrazones were found to inhibit soybean lipoxygenase type 1, a seed-derived enzyme also involved in lipid peroxidation reactions [5]. Gallaird and coworkers isolated lipoxygenase from cucumber fruit and showed that it was the initiator enzyme in a multi-component enzyme system capable of producing volatile compounds from long chain unsaturated fatty acids [6, 7]. The fatty acid hydroperoxides formed by lipoxygenase were cleaved by hydroperoxide lyase to produce volatile aldehydes. The cucumber-fruit-derived lipoxygenase–lyase system produced mainly volatile C_9 compounds whereas that from tea leaves produced volatile C_6 compounds [8].

Approximately 35 volatile compounds from wheat plants were isolated and identified [9–11] as part of a study of the interaction of wheat with fungal pathogens which cause rust disease. Many were thought to be formed through the combined action of lipoxygenase and hydroperoxide lyase. An investigation was made on the effects of some known inhibitors of lipoxygenase and cyclooxygenase on the generation of wheat plant volatiles [12]. Volatile compounds have been shown to promote germination of spores [13] and to influence differentiation and growth [14] of the fungal pathogens which cause rusts.

In the present study a comparison was made of the effects of three relatively potent inhibitors of lipoxygenase on wheat volatiles thought to be formed via lipoxygenase action. Subsequently, experiments were undertaken on tissue extracts to determine if an inhibitor, acetonylacetone *bis*-phenylhydrazone, would block the enzyme activity *in vitro*. This information was used to help confirm that the action of the inhibitor was on lipoxygenase *per se*. The effects of substrate addition to the homogenate and heat denaturation of enzymes on the volatiles formed by wheat plants were also evaluated.

RESULTS AND DISCUSSION

Acetonylacetone *bis*-phenylhydrazone (AABPH) was synthesized and purified by a method previously described [5]. The inhibitor was dissolved in methanol and then added to water prior to homogenization of fresh wheat plants. Volatiles were isolated by reduced pressure steam distillation–extraction, separated on a fused silica capillary GC column and quantitated relative to hexamethylbenzene.

The effect of AABPH on the generation of several volatile compounds from wheat plants is shown in Table 1. Compounds listed comprised *ca* 90% of the total yield of volatiles (60 ppm) obtained from fresh young plant homogenates by steam distillation–hexane extraction. The most marked change was the reduction in amount of 2-hexenal, which was the principal wheat volatile. The quantities of other C_6 compounds were also reduced following treatment with the inhibitor. The maximum inhibition of C_6 volatile production was observed at AABPH concentrations of *ca* 40 μM and above.

Table 1. Effect of acetonylacetone *bis*-phenylhydrazone (AABPH) on production of volatiles by wheat plants

Compound	Control	1	5	10	20	40	60	80	100
					μM of AABPH				
					Area units of compound*				
Hexanal	2370	1290	649	189	172	174	342	375	571
t-2-Hexenal	103000	86200	36400	23300	11600	4960	8410	6590	8080
1-Hexanol	947	600	385	197	356	170	137	128	167
t-2-Hexen-1-ol	5410	6000	3190	4250	2900	1010	968	1026	860
c-3-Hexen-1-ol	11100	10100	2190	2790	3810	2120	1390	2470	1270
Nonanal	449	465	385	474	339	282	528	406	268
t-2-Nonenal	133	88	108	88	95	114	132	119	132
t,c-2,6-Nonadienal	119	88	119	155	113	179	212	215	268
β-Ionone	1390	1350	1620	752	650	823	1220	1070	1440

*Normalized to GC peak area units of hexamethylbenzene.

Based on biosynthesis studies with leaves, Hatanaka *et al.* [15] presented a reaction scheme showing the formation of C_6 compounds through the action of a lipoxygenase on the C_{12}–C_{13} bonds of linoleic (18:2) and linolenic (18:3) acids. The lipoxygenase, in conjunction with a lyase, resulted in the cleavage of 18:2 to form hexanal and 18:3 to form *cis*-3-hexenal. The latter was relatively unstable and converted via an isomerase to *trans*-2-hexenal. The C_6 alcohols are believed to be formed by an alcohol dehydrogenase-mediated reduction of the corresponding aldehydes.

The amounts of other volatiles which constituted a much smaller fraction of the wheat constituents than the C_6 compounds did not consistently or clearly change as a result of treatment with AABPH. Of these, 2-nonenal and 2,6-nonadienal were generated in cucumber fruit via the cleavage of the C_9–C_{10} bonds of 18:2 and 18:3 [16]. The observation that there was not a marked reduction in these C_9 compounds indicates that there may be different lipoxygenases or lipoxygenase–hydroperoxide lyase enzyme systems involved in the formation of C_6 and C_9 compounds in wheat. Another possible explanation is that lesser depletions of C_{18} unsaturated fatty acids that are precursors of C_6 and C_9 compounds result in more C_9 precursors being available for C_9 compound formations. More definitive results could probably be obtained using plant tissues, such as cucumber, which generate C_9 compounds as their principal volatile components.

The origins of nonanal and β-ionone are not known although it was proposed that the former compound was formed from oleic acid (18:1) by autoxidation [17]. Based on structural similarities β-ionone may be formed from β-carotene.

Experiments were conducted on two other compounds, phenidone and nordihydroguaiaretic acid (NDGA), which are also reported as inhibitors of lipoxygenase activity [18, 19] (Table 2). The results were examined primarily to determine the effects of the inhibitors on the major volatiles such as 2-hexenal which were markedly reduced by AABPH. The two inhibitors were active but did not reduce the quantities of the major volatiles as effectively as AABPH. NDGA appears to be effective in reducing the amounts of unsaturated C_9 compounds. The phenolic nature of NDGA may result in less selective protein binding than the other inhibitors tested and contribute to the inhibition of both C_6 and C_9 compounds.

Knippel *et al.* [20] obtained evidence that phenidone and a ketone hydrazone, acetonephenylhydrazone (same class as AABPH), have different sites of action on soybean seed lipoxygenase. They showed that preincubation of enzyme with sulphydryl agents such as glutathione reduced the inhibitory potency of phenidone, whereas the same compound did not affect inhibition by acetone phenylhydrazone. However, the mode of action of these inhibitors remains to be established.

To determine whether enzyme denaturation would affect the generation of volatiles in wheat, boiling water was poured over harvested plant material and immersion was continued for 30 min before homogenation. The amounts of volatiles including lipoxygenase products were reduced drastically. The small quantities of each component that remained may have resulted partly from the generation of compounds when the plants were cut at harvest. Interestingly, virtually all volatile compounds that were observed in GC traces, including compounds thought to be formed by autoxidation (for example, nonanal), were diminished upon hot water treatment. Based on this result it was concluded that enzymatic products were needed for autoxidation to proceed extensively in wheat plants. Perhaps lipase enzymes are required to release bound fatty acids prior to chemical oxidation.

To determine the effect of added substrate on the generation of volatiles, plants were homogenized with an aqueous solution of sodium 18:2 prior to the isolation of compounds. Relatively large amounts of hexanal, its corresponding alcohol, 1-hexanol, and 2-nonenal were generated compared to controls without added fatty acid salt (Table 3). These were the volatile compounds which would be expected to be most affected by the action of a lipoxygenase–hydroperoxide lyase type enzyme system on 18:2 as discussed above. In addition, there was a very large increase in *trans, trans*-2,4-decadienal. A trace quantity of this compound was detected earlier in wheat by GC-MS analysis [unpublished results]. The mechanism for formation of this compound has not been established.

Results on the composition of fatty acids in wheat which are the presumed precursors for many of the volatile compounds formed by the plant are given in

Table 2. Effects of phenidone and nordihydroguaiaretic acid (NDGA) on production of volatiles by wheat plants

Compound	Control	μM of Phenidone 60	100	200	μM of NDGA 100	200	300
	Area units of compound*						
Hexanal	3650	1230	647	351	1030	1990	299
t-2-Hexenal	73800	60900	44000	24600	81900	61100	23000
1-Hexanol	1330	449	334	228	393	210	112
t-2-Hexen-1-ol	4370	5030	3500	2310	3220	2270	1150
c-3-Hexen-1-ol	5310	2540	2580	1860	5360	3810	2250
Nonanal	305	553	330	306	218	243	353
t-2-Nonenal	159	157	142	123	65	42	36
t,c-2,6-Nonadienal	112	150	126	117	48	42	46
β-Ionone	1500	1400	1130	733	838	608	384

*Normalized to GC peak area units of hexamethylbenzene.

Table 3. Effect of the addition of linoleate on the synthesis of volatile compounds by wheat plants

Compound	Plant control	Linoleate + plant	Linoleate
	Area units of compound*		
Hexanal	4440	99500	2430
t-2-Hexenal	85400	190000	—
1-Hexanol	2430	11200	—
t-2-Hexen-1-ol	5640	3450	—
c-3-Hexen-1-ol	10600	6660	—
Nonanal	1500	1370	—
t-2-Nonenal	205	1390	85
t,c-2,6-Nonadienal	130	270	—
β-Ionone	1650	2250	—
t,t-2,4-Decadienal	178	32900	106

*Normalized to GC peak area units of hexamethylbenzene; means of two determinations except linoleate column.

Table 4. 18:3 and 18:2 comprised 80% of the fatty acids of wheat. Our results with wheat volatiles were consistent with our determined amounts of fatty acid substrate in the plants. For example, the aldehyde derived from 18:3, that is 2-hexenal, was the principal volatile whereas 18:3 was the major fatty acid found in the plants. Hexanal also occurred in relatively large amounts as did its precursor 18:2. It is notable that the greenhouse grown plants used in the present studies resembled the immature field grown plants in previous studies [11] in that they produced large quantities of C_6 compounds relative to other volatiles. On a fr. wt. basis the total quantity of fatty acids accounted for 0.48% of the plant composition. Since the total yield of volatiles per fr. wt in the present study was 0.006% (60 ppm), the quantity of fatty acids converted to volatiles was very small.

The other fatty acids found in wheat do not serve as substrates for lipoxygenase because they do not have the required *cis,cis*-1,4-pentadiene structure. It is interesting that palmitic acid (16:0) is a major component and pentadecanal, a compound which is thought to be the principal α-oxidation product of 16:0 was found as a relatively major component of wheat volatiles earlier [10].

To help confirm that the inhibitor AABPH acted on wheat lipoxygenase, an experiment was undertaken to prepare a wheat plant extract with lipoxygenase activity. Extraction procedures including addition of protease inhibitors resulted in a preparation with lipoxygenase activity which was capable of forming the hydroperoxide of linoleic acid in a standard assay procedure as shown by substrate dependent absorbance change at 235 nm [21]. Wheat plant extracts also displayed 18:2 dependent O_2 consumption (data not shown) and contain a polypeptide of M_r *ca* 26000 which cross-reacts with soybean seed lipoxygenase antibodies on Western blots [22]. The results of experiments conducted with AABPH over a range of concentrations (Fig. 1) indicated that enzyme activity was completely inhibited at 2.5 μM AABPH. There was a narrow range of AABPH inhibition with minimal loss of activity at concentrations of 0.5 μM AABPH or less. Results with soybean seed lipoxygenase [5] showed that the IC_{50} for AABPH was 0.15–0.2 μM which was lower than that for the wheat system. However, the soybean enzyme was a highly purified enzyme and, in

Table 4. Fatty acid composition of wheat plants

Fatty acid	Percent of total fatty acids*
16:0	13.8
16:1	3.8
18:1	1.8
18:2	11.7
18:3	68.7

*Fatty acids comprised 0.48% of plant fr. wt; mean of two determinations.

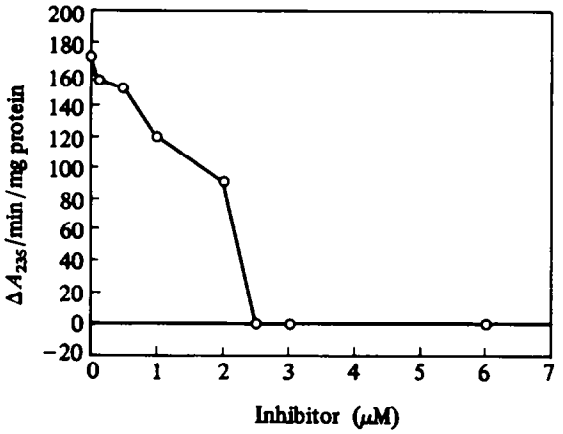

Fig. 1. Effect of AABPH concentration on the lipoxygenase activity of an extract prepared from wheat plants.

addition, was type 1 lipoxygenase active at pH 8 whereas the wheat enzyme was probably type 2 since it had much higher activity at pH 6.8 than at pH 8. Type 2 lipoxygenase also appears to predominate in soybean leaves [Hildebrand, unpublished results].

Josephson *et al.* [23] recently published work on the inhibition of fish volatile biogenesis using aspirin and stannous chloride (cyclooxygenase and lipoxygenase inhibitors, respectively). They found that these compounds inhibited the formation of most volatile carbonyls and alcohols. That is, the compounds were minimally selective in their inhibition of volatile compounds within a class at the concentrations used (0.01 M for lipoxygenase inhibitor). This contrasts to the C_6 compound selectivity exhibited by the inhibitors that we tested on wheat lipoxygenase.

Wheat plant homogenates synthesize a wide array of volatile compounds. Principal among these are C_6 volatile aldehydes and alcohols which derive from the action of lipoxygenase on long-chain, unsaturated fatty acids, namely 18:2 and 18:3. Our results demonstrate that a ketone hydrazone, acetonylacetone *bis*-phenylhydrazone, is a potent inhibitor of the synthesis of these C_6 compounds in wheat. This compound blocks the action of a wheat extract with lipoxygenase activity *in vitro* at low concentrations (2.5 μM). Addition of 18:2 to wheat increased the quantities of predicted lipoxygenase-derived volatile products as well as 2,4-decadienal. Heat treatment of plants greatly decreased the quantities of lipoxygenase-derived products and most other volatiles generated by wheat indicating the need for enzyme activity in the formation of these compounds. The C_6 compounds formed by wheat occur widely, especially in leaves, and two of the compounds, *trans*-2-hexenal and *cis*-3-hexen-1-ol, are known as leaf aldehyde and leaf alcohol. With regard to possible biological functions of C_6 compounds, Grambow [14] showed that *cis*-3-hexen-1-ol, *trans*-2-hexenal and other volatile compounds tested *in vitro* stimulated the differentiation of infection structures and inhibited growth of the wheat rust fungus. Volatile C_9 compounds, such as nonanal, have been shown to promote fungal spore germination of wheat rust [13]. It has also been proposed that some of the compounds resulting from lipoxygenase action in wounded plant tissue may be involved in induced pest resistance [24]. Based on the above results it would be interesting in future experiments to treat wheat plants with AABPH solution (dip or spray application) to determine whether the compound influences infection by rust fungal pathogens.

EXPERIMENTAL

Plant material. Winter wheat plants 'Arthur 71' and 'Caldwell' were grown in the soil floor of a greenhouse from February to June, 1986. They were cut at the soil surface and used fresh for all expts. Plants at harvest ranged from 25 to 50 cm tall.

Preparation of inhibitor solutions and treatment of plant tissue. Phenidone and nordihydroguaiaretic acid were obtained from Aldrich. Acetonylacetone *bis*-phenylhydrazone was synthesized [5] and kept in an evacuated desiccator in a freezer except during weighing. Solns of inhibitors (4 l. each and 0–300 μM) were freshly prepared as follows. A weighed amount of compound was dissolved in 3 ml of MeOH and the resultant soln slowly injected beneath the liquid surface into 4 l. of H_2O with continuous vigorous stirring. The H_2O had been purged with N_2 for 30 min just before the addition of inhibitor. The solns were then aerated for 30 min and used immediately to evaluate the effect of the inhibitors on volatile compound generation. Fresh wheat plants (100 g) were homogenized with 1 l. of inhibitor soln in a Waring blender at medium speed for 1 min. The homogenate and the remaining 3 l. of inhibitor soln were combined and subjected to steam distillation–extraction to isolate the volatiles as described below.

Control expts were also carried out according to the procedures outlined above and included an equivalent amount of MeOH.

Heat denaturation of enzymes. Boiling H_2O (1.5 l.) was poured over 100 g of wheat and the plants immersed in the water for 30 min. The plants and water were then homogenized and combined with 2.5 l. of H_2O. The volatiles were isolated as described below.

Treatment with sodium linoleate. Na 18:2 (Sigma) (1 g) was dissolved with stirring in 1 l. of distilled H_2O, added to 100 g of wheat and the mixture then homogenized. Three more l. of H_2O were then added to the homogenate and the volatiles isolated as described below.

Isolation of volatiles by steam distillation–extraction. Aqueous homogenate (4 l.) was placed in a 12 l. flask equipped with a Likens-Nickerson distillation–extraction apparatus [25] with 40 ml of redistilled hexane in the solvent reservoir. After heating the distillation flask to *ca* 65° (over a 1 hr period) and lowering

the condenser coolant to 5° the pressure in the apparatus was reduced to *ca* 220 mmHg and distillation–extraction carried out for 3 hr. The hexane extract was then removed and concd to *ca* 1 ml using a micro-distillation apparatus and a Vigreaux column.

Capillary GC analysis of volatile compounds. Hexamethylbenzene (20 μg), was added to the hexane soln as int. std and an aliquot was chromatographed on a 60 m × 0.32 mm Supelcowax 10 fused silica capillary column. The column was held isothermal at 60° for 1 min and then programmed at 3°/min to 220°. Compound identification was confirmed by co-elution with authentic samples [9, 10] purchased commercially or gifts from Bedoukian Research, Inc., Danbury, CT. GC peak areas for compounds were integrated electronically and normalized to the int. std. At least two runs were made for the heat denaturation treatment, the Na 18:2 expt and controls, and the mean values for the compounds are reported.

AABPH at higher concentrations yielded a peak during distillation–extraction which interfered with the quantitation of 2-nonenal on the Supelcowax column. This component, however, was resolved from 2-nonenal on a 60 m × 0.25 mm SE-54 column. 2-Nonenal was quantitated using peak ht measurements obtained from these chromatograms.

Fatty acid analysis. Lipids were extracted from 100 mg lyophilized wheat plant tissue with 4 ml of $CHCl_3$–MeOH (2:1) [26]. One millilitre of 1% H_2SO_4 in MeOH was added and the sample heated to 70° for 10 min. The temp. was raised to 90° and maintained at this level until the sample vol. was reduced to 0.5 ml. The sample was cooled to room temp. before addition of 2 ml of petrol containing 0.01% butylated hydroxytoluene. The organic phase containing the resulting fatty acid Me esters was removed and dried (Na_2SO_4). An aliquot was analysed by GC on 3% SP-2310/2% SP-2300 on Chromosorb W. The instrument was operated isothermally at 170° for 2 min and then programmed at 5°/min to 210°. Peaks were identified and quantitated using authentic standards.

Preparation of wheat extract with lipoxygenase activity and assay of AABPH inhibition. Wheat plants were collected on ice and frozen in liquid N_2. One gram quantities of tissue were ground in a mortar and pestle together with liquid N_2 and 25 mg of PVP. Two millilitres of the following extraction buffer was then added to the powdered wheat tissue before further grinding of the material: 0.35 M sucrose, 25 mM HEPES (pH 7.5), 1% octylglucosylpyranoside, 1% BSA, 0.1% Na ascorbate, 0.05% thimersol, and the protease inhibitors: 1 mM phenylmethylsulphonyl fluoride, 1 mM *e*-aminocaproic acid, 1 mM benzamidine–HCl and 10 μM leupeptin. The homogenate was centrifuged × 2 at 12000 *g* for 20 min and the second supernatant collected and used for the lipoxygenase assay.

Lipoxygenase activities of wheat extracts were determined at 23° by a modification of the standard spectrophotometric assay [21]. The substrate contained 3.2 mM KOH, 0.05 M Na Pi (pH 6.8), 0.16% Tween 20, 0.002% Na azide, and 1.3 μM 18:2. Aliquots of the wheat extract were added to the substrate containing 0–60 μM AABPH added as a soln in Me_2CO (the solvent had no effect on lipoxygenase activity at the levels used in these expts). Lipoxygenase activity was calculated from the change in *A* at 235 nm per mg protein using a double beam spectrophotometer. The protein content of the wheat extracts was determined using the Biorad protein assay based on the method of ref. [27].

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