Lipoxygenase 3 Reduces Hexanal Production from Soybean Seed Homogenates[†]

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Hexanal is a volatile aldehyde formed from disrupted soybean seeds such as during processing. This compound has an undesirable aroma that has limited some uses of soybean proteins in food products. Lipoxygenase, which oxidizes linoleic acid and leads to hexanal formation, exists in soybean seeds as three isozymes. It was thought that all three lipoxygenase isozymes contribute to hexanal production with isozyme 2 being the most effective. Our studies with near isogenic soybean lines deficient in the various lipoxygenase isozymes indicate that lipoxygenase 3 reduces hexanal formation in aqueous homogenates of soybean seed. Experiments with purified enzyme confirm that lipoxygenase 3 can decrease hexanal yield. Preliminary results suggest that lipoxygenase 3 reduces hexanal formation by converting the lipoxygenase product, 13-hydroperoxy-9,11-octadecadienoic acid, into forms unavailable for conversion to hexanal. The different effects of lipoxygenase isozymes on the generation of hexanal have implications from the standpoints of food uses of plant products and in the physiological roles of particular lipoxygenase isozymes.

INTRODUCTION

Hexanal has a low olfactory threshold (Buttery et al., 1969) and an objectionable odor that may impart an undesirable flavor to food products. This compound is formed by hydroperoxidation of linoleic acid (*cis,cis*-9,12-octadecadienoic acid) in plant tissues through the action of lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) and subsequent cleavage of the product by hydroperoxide lyase (Hatanaka et al., 1987). Relatively high levels of both linoleate and lipoxygenase and organoleptically unacceptable amounts of hexanal are produced by soybeans (*Glycine max* L. Merr.) (Arai et al., 1970; Axelrod et al., 1981; Rackis et al., 1979; Wolf, 1975). This has limited the use of whole soybeans and soybean protein in certain food products. Seeds of commercial soybean cultivars contain three distinct lipoxygenase isozymes designated lipoxygenases 1, 2, and 3, and these isozymes were thought to contribute to hexanal formation (Arai et al., 1970; Axelrod et al., 1981; Rackis et al., 1979; Wolf, 1975). Soybeans have been screened for mutants without seed lipoxygenase(s), and lines with nondetectable or very low amounts of seed lipoxygenase isozymes have been found (Hildebrand and Hymowitz, 1981; Kitamura, 1984). These lines, referred to as null mutants, were inherited as simple recessive alleles. Lipoxygenases 1 and 2 were tightly linked, but lipoxygenase 3 was inherited independently of lipoxygenases 1 and 2 (Davies and Nielsen, 1986). Davies and Nielsen (1987) developed near-isogenic lines backcrossed to the soybean cultivar Century that are homozygous for one or two of the lipoxygenase null alleles. No triple null lines have been found because of the tight linkage of lipoxygenases 1 and 2 and possible lethality of low-frequency recombinants.

Matoba et al. (1985) examined the generation of hexanal by aqueous seed homogenates of the original lipoxygenase null mutants and concluded that lipoxygenase 2 was largely responsible for the generation of hexanal. Davies et al. (1987) also concluded that elimination of lipoxygenase 2 through genetic selection was important in improving the flavor of soybean preparations. However, it became apparent in the course of our studies of the lipoxygenase null backcross lines that a major effect on hexanal production was due to the presence or absence of lipoxygenase 3 and that this effect differed from that expected for a lipoxygenase.

MATERIALS AND METHODS

Hexanal Production of Lipoxygenase Mutant Isolines. Hexanal production was analyzed by using aqueous homogenates of lyophilized, powdered meal from mature soybean seeds of the backcross lipoxygenase mutant lines (Davies and Nielsen, 1987) and the wild-type recurrent parent Century. The genotypes designated –L2L3, –L1L3, –L2, –L3, and –L1, kindly provided by Dr. Niels Nielsen, Purdue University, are lines backcrossed to Century that are homozygous recessive for the null (or very low) alleles indicated (Davies and Nielsen, 1987). For example, –L2L3 is a null for lipoxygenases 2 and 3 (but has the normal wild-type level of lipoxygenase 1). Century seeds contain lipoxygenases 1–3 (as do all widely grown commercial soybean cultivars). These analyses were performed on seeds harvested from plants grown concomitantly in a greenhouse with illumination supplemented to 13 h with high-intensity sodium halide lamps.

The hexanal production analyses were performed in 1.8-mL screwtop glass vials. To each vial was added 10 mg of seed meal, 50 μL of 0.1 M sodium phosphate, pH 6.8, and 150 μL of 1 mM sodium linoleate containing 15 μg of cyclohexanone as an internal standard. The samples were capped and stirred by using a magnetic stir bar for 30 s and then incubated in a water bath at 30 °C for 30 min, after which time 250-μL headspace vapor samples were withdrawn by using a gastight syringe. The samples were immediately analyzed by gas chromatography using direct injection onto a Varian 3700 gas chromatograph containing a 30 m × 0.53 mm DB-5 (methylsilicone) fused silica column.

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The GC conditions were as follows: injector 220 °C; FID 240 °C; column oven isothermal at 50 °C for 1 min and then temperature programmed at 5 °C/min to 200 °C; He carrier flow rate 6 mL min⁻¹. The identification of hexanal was confirmed by mass spectral analysis obtained with a Hewlett-Packard 5985A instrument and cochromatography on the DB-5 column. The results from GC quantification are presented as nanograms of hexanal per milliliter of headspace relative to the cyclohexanone internal standard. The GC response factor for *n*-hexanal was determined in the vapor phase as recommended by the U.S. EPA by using a 2-L static dilution bottle (Tekmar Co.). The data presented represent means of three analyses ± standard errors (SE).

Purification of Lipoxygenase 3. Lipoxygenase 3 was purified from the -L2 line by the following procedure: Seed meal (1 g) was extracted on ice with 15 mL of 0.02 M sodium phosphate, pH 6.83, by using a mortar and pestle, filtered through miracloth (Calbiochem), and centrifuged at 12000*g* for 10 min at 4 °C. Calcium chloride (70 μM) was added to the supernatant, which was then incubated for 30 min on ice. The preparation was then centrifuged as above, and the resulting supernatant was chromatographed on a Sephadex G-50 column (2.5 × 30 cm) using the same buffer as for extraction. The lipoxygenase peak was separated into lipoxygenases 1 and 3 on a PBE 94 chromatofocusing column (Pharmacia, Inc.) (2.5 × 30 cm) as described by Funk et al. (1985). Lipoxygenase 3 was further purified by using a DEAE-Sephadex A-50 column which was eluted with a linear gradient of 0.02–0.2 M sodium phosphate, pH 6.8, followed by gel filtration again on Sephadex G-50 as above. The purified lipoxygenase 3 was concentrated by using a Centricon 30 microconcentrator (Amicon Co.). This lipoxygenase 3 preparation gave only one band on native IEF (pH 4–7) and a band at 97 000 (the molecular weight for lipoxygenase 3) accounting for ca. 95% of the coomassie staining of SDS-PAGE gels (a second band with a lower molecular weight was immunodecorated with lipoxygenase 3 antibodies, indicating that it was probably a lipoxygenase 3 degradation product).

Addition of Lipoxygenase 3 to Homogenates. Reaction conditions for the addition of purified lipoxygenase 3 to soybean homogenates were the same as described for the mutant isolines above with the following exception: 50 μL of a solution of purified lipoxygenase 3 in 0.1 M sodium phosphate, pH 6.8 [543 μkat μL⁻¹ determined spectrophotometrically by using 23 000 as the molar extinction coefficient for the hydroperoxide reaction product (Gibian and Vandenberg, 1987)] was added to the reaction mixture in place of 50 μL of 0.1 M sodium phosphate described above. This quantity of lipoxygenase 3 was approximately the same as the endogenous level in wild-type seeds. This study was performed with the backcross mutant isolines (Davies and Nielsen, 1987) -L2L3, -L1L3, and -L3 and the wild-type recurrent parent Century.

The dosage response of lipoxygenase 3 addition on hexanal production was also determined. Hexanal production [ng of hexanal (mL of headspace)⁻¹] with increasing units [μkat (mg of protein)⁻¹] of lipoxygenase 3 addition was established under conditions as above with the -L3 isoline.

Hexanal Formation with Linoleic Acid versus Its Hydroperoxide Derivative. The formation of hexanal of the lipoxygenase 3 null line (-L3) was compared with that of Century in the presence of 1 mM linoleic acid (18:2) and 1 mM 13-hydroxyperoxy-9,11-octadecadienoic acid (HOO18:2). HOO18:2 was prepared by using 18:2 and soybean lipoxygenase as described by Gardner (1975) except that the reaction was carried out at 0 °C as suggested by Gardner (personal communication).

RESULTS AND DISCUSSION

All lines null for lipoxygenase 3 yielded considerably greater quantities of hexanal than those with lipoxygenase 3 (Figure 1). Similar trends were seen for seed homogenates without added linoleate, but the results were more variable apparently due to differences in endogenous levels of free fatty acid substrate (linoleic acid or 18:2), which was found to be correlated with hexanal
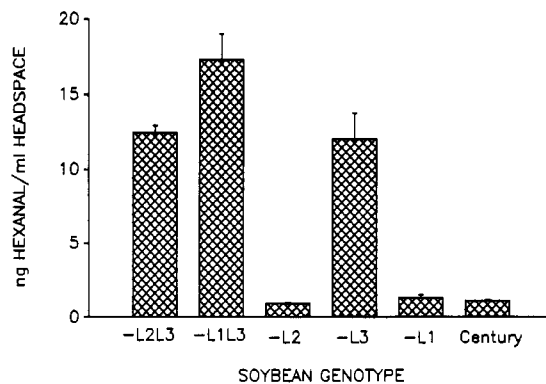

Figure 1. Hexanal production by aqueous homogenates from mature soybean seeds of the backcross lipoxygenase mutant lines (Davies and Nielsen, 1987) and the wild-type recurrent parent Century (see Materials and Methods). Genotypes -L2L3 and -L1L3 refer to double-mutant isolines deficient in lipoxygenases 2 and 3 and 1 and 3, respectively. -L2, -L3, and -L1 refer to lines deficient in lipoxygenases 2, 3, and 1, respectively. Century, like all major commercial soybean cultivars, has high levels of lipoxygenases 1–3.

Table I. Effect of Lipoxygenase 3 Addition (27 mkat) to Soybean Homogenates on Yield of Hexanal

treatment	ng of hexanal (mL of headspace)⁻¹			
	-L2L3	-L1L3	-L3	Century
control	12.6 ± 0.3ᵃ	17.3 ± 1.0	12.1 ± 1.0	1.1 ± 0.6
LOX 3	0.74 ± 0.06	0.80 ± 0.06	0.98 ± 0.6	0.6 ± 0.12

ᵃ Mean of three replications ± standard errors.

Table II. Hexanal Formation of the Lipoxygenase 3 Null Line (-L3) Compared with That of Century (+L3) in the Presence of 1 mM Linoleic Acid (18:2) and 1 mM 13-Hydroperoxy-9,11-octadecadienoic Acid (HOO18:2)

genotype	ng of hexanal (mL of headspace)⁻¹	
	18:2	HOO18:2
Century	1.22ᵃ	7.5
-L3	20.0	13.6

ᵃ All means shown are significantly different at the 1% level (Fischer's least significant difference, 5.65).

production (data not shown). Hexanal was not detected from homogenates of autoclaved meal of wild-type soybeans, cultivar Century, incubated under conditions as in Figure 1. Lipoxygenase 3 was subsequently purified and added to soybean seed homogenates. These additions resulted in an approximately 10–20-fold reduction in hexanal yield in the lines that were homozygous recessive for the lipoxygenase 3 null allele and a nearly 2-fold reduction in hexanal from Century (which had relatively high levels of lipoxygenase 3) (Table I). The quantity of enzyme used in these experiments [27 mkat] was similar to that detected in 10 mg of Century seed meal. Additions of lipoxygenase 3 reduced hexanal production in a dose-dependent manner (Figure 2). Additions of autoclaved lipoxygenase 3 had virtually no effect on hexanal yield, indicating that an active enzyme is needed to reduce hexanal formation. This is consistent with a report of Rackis et al. (1979) that hexanal production in many plant seeds, including soybeans, requires active lipoxygenase and that autoxidation of fatty acid substrate does not contribute greatly to hexanal formation in aqueous preparations from seeds.

Lipoxygenase 3 preparations have been reported to catalyze reactions with substrate in which nonvolatile ketodienes are formed (Axelrod et al., 1981; Schewe et al., 1986). Thus, lipoxygenase 3 may reduce hexanal yield in soybean seed by competing with lipoxygenases 2 and 1 for
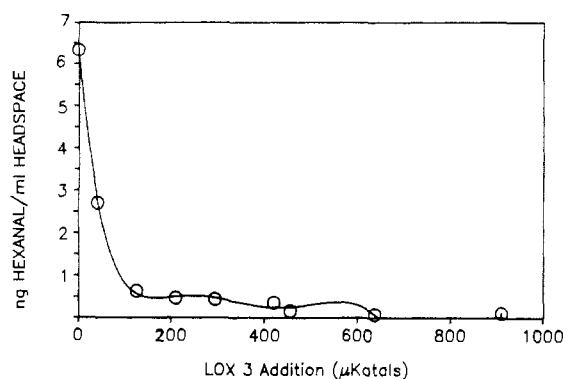
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Figure 2. Dosage response of lipoxygenase 3 addition on hexanal production of the -L3 mutant isoline. The equation of the curve is $P(X) = 6.2477\,44 \times 10^4 - 8.1311 \times 10^2 X + 4.4116 X^2 - 1.1733 \times 10^{-2} X^3 + 1.6127 \times 10^{-5} X^4 - 1.0932 \times 10^{-8} X^5 + 2.8682 \times 10^{-12} X^6$, and the R value is 0.99952. The reaction conditions were as under Materials and Methods.

the available fatty acid or fatty acid hydroperoxide. Studies were therefore conducted to examine hexanal formation in soybean seed homogenates with and without lipoxygenase 3 in the presence of 1 mM 18:2 or 1 mM 13-hydroxyperoxy-9,11-octadecadienoic acid (HOO18:2). A possible mechanism by which lipoxygenase 3 reduces hexanal formation could be by converting HOO18:2 into products that are not substrates for hexanal formation by hydroperoxide lyase. As the HOO18:2 is formed from 18:2, the lipoxygenase 3 would compete with the hydroperoxide lyase. If HOO18:2 were added instead of 18:2, however, hydroperoxide substrate would be less limiting for hexanal formation. Hexanal yield of soybean homogenates was more than 16 times higher in the absence of lipoxygenase 3 with 18:2 but only about 2 times higher with HOO18:2 (Table II). Thus, these preliminary studies indicate that lipoxygenase 3 is converting the HOO18:2 formed by lipoxygenases 1-3 into products unavailable to hydroperoxide lyase. Further studies will need to be conducted to determine the precise chemical nature of the product(s) of lipoxygenase 3 and HOO18:2.

CONCLUSIONS

These experiments demonstrate that lipoxygenase 3 reduces the yield of hexanal in soybean seed homogenates. This reduction is dependent on the dose of active lipoxygenase 3. Lipoxygenase 3 apparently reduces hexanal production by converting hydroperoxylinoleic acid into products unavailable to the hyperoxide lyase reaction.

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