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Evidence of an Enzymatic Source of Off Flavors in "Lipoxygenase-Null" Soybeans

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Abstract The utilization of soybean products as food ingredients and foods is often limited by their beany-grassy flavor. Eliminating seed lipoxygenase (LOX) isozymes 1, 2 and 3 reduces the amounts of volatile off-flavor compounds in stored soybeans and soy products significantly, but they are not completely eliminated. The present work presents evidence that lipoxygenase-null (LOX-null) soybeans contain a LOX-like enzyme that is responsible for the off-flavors in LOX-null soybeans. Volatiles production in triple LOX-null soybeans was terminated by heat treatment, which suggests an enzymatic cause to the off-flavors. The source is LOX-like in that the volatile compounds produced are similar to LOX-generated products of polyunsaturated fatty acids. Oxygen was consumed when a LOX-null protein solution was incubated with crude soybean oil suggesting that the enzyme catalyzed oxygen consuming reactions. The generation of flavor compounds was inhibited by the typical LOX inhibitors propyl gallate and nordihydroguaiaretic acid (NDGA). The enzyme appears to be more active with phosphatidylcholine than with other lipid substrates. The cause of the off-flavors in LOX-null beans appears to have enzyme-like characteristics. This is the first report of the initial characterization of this LOX-like enzyme.

Keywords Lipoxygenase-like enzyme · Flavor · Lipoxygenase · Enzyme · Soybeans · Volatile off-flavor compounds · Phosphatidylcholine · Lipoxygenase inhibitors

Abbreviations

LOX	Lipoxygenase enzyme
SPME	Solid phase microextraction
PC	Phosphatidyl choline
PI	Phosphatidyl inositol
PE	Phosphatidyl ethanolamine
NDGA	Nordihydroguaiaretic acid
PG	Propyl gallate
FFA	Free fatty acid

Introduction

The sale of food products made from soybeans has grown dramatically over the last 15 years. A variety of health claims for soy protein and soymilk have driven this increase in sales. Sales of soybean products have increased from $300 million in 1992 to nearly $4 billion in 2004. Soymilk has led the sector with a 14% increase between 2003 and 2004 alone [1]. However, there was only a 1.4% increase in total soy food sales and 7.2% increase in soymilk sales between 2005 and 2006 [2]. Currently available soy and soymilk products continue to exhibit flavor and textural problems.

The predominant off-flavors in these products come from the activity of endogenous lipoxygenase enzymes

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(LOX) found in soybeans. The off-flavor compounds, which derive from lipid oxidation initiated by these enzymes, are described as "painty, bean, green and unpleasant". While processing methods for soymilk have improved and reduced the off-flavors, soybean products still often have undesirable chalky texture caused by processing. It was anticipated that developing soybeans that were free from the three known seed LOX enzymes would provide a solution to soybean flavor problems. Cultivars of LOX-null soybeans do show reduced LOX-derived aromas, however, these beans are not completely free from typical LOX-derived flavor compounds. In studies conducted by NASA, LOX-null beans have been shown to develop the characteristic off-flavors associated with LOX (Wilson L. A. personal communication) and these observations have caused concern for use of soybeans in long-term space expeditions.

Lipoxygenases (EC 1.13.11.-) are a class dioxygenases containing non-heme iron. Lipoxygenases catalyze dioxygenation of polyunsaturated fatty acids containing a *cis*, *cis*-(1*Z*, 4*Z*) pentadiene system and form conjugated hydroperoxydienes. Linoleate and linolenate esters in triacylglycerols are the most important LOX substrates in soybeans. The predominant hydroperoxides formed are at the C-9 and C-13 positions on the fatty acid chain resulting in, from linoleic acid, 9(Z),11(E)-13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD) or 10(*S*)-12(*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) and from linolenic acid 10(E),12(Z),15(Z)-9-hydroperoxy-10,12-15-octadecatrienoic acid (9-HPOT) and 9(Z),11(E)15(Z)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT). These hydroperoxides are involved in further enzymatic reactions resulting in spontaneous or enzymatically caused cleavage of the fatty acid chain. When this occurs, aldehydes (hexanal, nonenal) and ketones are released causing the off flavor that is characteristic of soybeans.

At least six LOX isozymes have been found in soybean plants. These are identified as LOX 1-6; LOX-1, LOX-2 and LOX-3 are storage proteins found in dormant soybean seeds and are each a product of specific genes. The remaining LOX isozymes are found only in plant tissue and are believed to be part of the plant defense mechanism [7]. The first three isozymes have been eliminated from LOX triple-null beans. The storage LOXs are known to contain an iron atom which is essential for LOX enzymatic activity. Glickman and Klinman [3] reported that free active LOX is in the ferric state and transformation from iron (II) to active iron (III) is hydroperoxide dependent.

Mutants of soybeans that are deficient in one of the LOX 1–3 genes have been reported throughout the literature. The goal of these breeding and mutational programs was to eliminate or substantially reduce the off-flavors associated with LOX activity. Eventually, stable and agronomically viable triple-null mutants were produced [4]; these soybeans were deficient in the LOX 1–3 genes. The LOX-null beans were shown to produce tofu and soymilk with improved flavor and sensory qualities [5, 6]. However, volatile aldehydes and ketones that result from LOX or auto-oxidation of polyunsaturated fatty acids have been found in the headspace of both LOX-null and conventional beans [7, 8]. The objective of the present research was to determine if these volatiles were derived from a LOX-like enzyme or spontaneously from autoxidation.

Materials and Methods

The soybean (*Glycine max*) varieties used were Century (conventional, positive control) and IA2025 (LOX triple-null). LOX-null soybeans were tested for the presence of residual lipoxygenases 1, 2 and 3 according to Hammond et al. [9]. The soybeans were cracked, dehulled and flaked in the pilot plant of the Center for Crops Utilization and Research (Iowa State University) and the flakes were extracted in a Soxhlet extractor with hexanes to remove lipids. The oil was separated from the solvent with a Buchi rotary evaporator, and the recovered oil was sparged with carbon dioxide to remove residual solvent. After hexane was evaporated from the defatted soy flakes, they were soaked with 0.1 M acetate buffer (9 g flakes per 91 g of buffer) for 2 h and the resulting soymilk was centrifuged at 800 × g for 5 min, and the soy extract supernatant was used in subsequent experiments.

Solid-Phase Micro-Extraction (SPME) and Gas Chromatographic Analysis

About 2 ml of soy extract or 0.2 g flakes were placed in a 150-ml glass SPME vessel and sealed. A 2-cm 50/30 μm DVB/Carboxen/PDMS StableFlex fiber was inserted through a Teflon seal to trap the volatile compounds. The sealed samples were held with stirring at 40 °C for 45 min. Then the fiber apparatus was removed from the bottle and inserted for 15 min into the injection port of an HP 5890 Series II gas chromatograph (Hewlett-Packard Company, Avondale, PA) fitted with a fused-silica capillary column SPB-1 (30 m × 0.25 mm i.d., 0.25 μm) (Supelco, Inc., Bellefonte, PA). The carrier gas (helium) flow rate was 1.9 ml/min, and the split ratio was 2.64. The oven temperature was held at 30 °C for 3 min, programmed to 100 °C at 4 °C/min and then to 220 °C at 15 °C /min and held at 220 °C for 5 min. The injector temperature was 220 °C, and the detector temperature was 250 °C. Peaks were identified by comparing their retention times and mass spectra with those of known compounds.

Mass Spectrometry

Electron impact GC/MS experiments were conducted using a Micromass GCT mass spectrometer (Premier, Waters, Sollentuna, Sweden) coupled to the Agilent 6890 GC System. The mass spectra were recorded in the mass range of 35–650. The carrier gas was helium and the flow rate was kept at 1.1 ml/min throughout the run. The head pressure was maintained constant at 10 psi throughout the run. The injector port and the interface were set at 220 and 260 °C, respectively. The same sample preparation method and GC conditions used for SPME were used, except that the GC column was an HP-5 (polydimethylsiloxane with 5% phenyl groups, fused silica open tubular column, 30 m × 0.25 mm i.d., 0.25 μm film thickness).

Dissolved Oxygen Analysis

Dissolved oxygen was measured by using a Broadley-James Dissolved Oxygen Transmitter at 25 °C. One gram of crude or refined soybean oil was dissolved in 25 ml of absolute ethanol; 3 ml of this solution were added to 100 ml of 0.1 M potassium monobasic phosphate buffer solution pH 7 along with 0.1 g of finely ground and defatted soybean flakes. The oxygen uptake of stirred samples was measured by calibrated probe for 24 h at 25 °C. Dissolved oxygen was read from the transmitter and recorded by hand; for the first hour readings were taken each minute, beyond an hour readings were taken every 3 h. After 18 h, the measurements did not change through the next 6 h and the termination of the experiment. Each analysis was done in triplicate.

Heat Experiments

In a SPME vessel, defatted soy flakes or their extracts were heated at 60 °C for 1–120 min in a water bath. Volatile compounds and dissolved oxygen were analyzed before and after heat treatment. Heating for 10 min at 60 °C was the minimum time needed to inactivate oxygen uptake in LOX-null and Century flakes.

Substrate Utilization

Soy extract was incubated with different substrates: soybean oil, soy lecithin (Fisher Scientific, Pittsburgh, PA), palmitoyl-linoleoyl phosphatidylcholine (PC), palmitoyl-linoleoyl phosphatidylethanolamine (PE) or palmitoyl-linoleoyl phosphatidylinositol (PI) (Avanti Polar Lipids, Inc, Alabaster, AL) and free fatty acids (FFAs) linoleic acid and linolenic acid at 40 °C for 45 min. Volatile compounds were analyzed by SPME GC.

Effect of LOX Inhibitors

Soy lecithin with 0.6 μM propyl gallate or with 200 μM nordihydroguaiaretic acid (NDGA) were added to soy extract and incubated for 18 h. As described above, volatile compounds were collected by SPME and analyzed by GC. Controls without LOX inhibitors were used for comparison.

Effect of pH

Soy extract was prepared in three buffered systems [12]: acetate buffer (for pH 3–5.5), potassium phosphate buffer (for pH 5.5–8) and borate buffer (for pH 8.0–10.0). Volatile products were measured as described by SPME GC.

Statistical Analysis

Data were analyzed by using Analysis of Variance (ANOVA) and Tukey's test to detect significant differences among the different treatments by SAS [version 9.1, SAS Institute Inc.] general linear model procedure. The level of significance was set at $\alpha = 0.05$. Two years of conventional and null-LOX soybeans (crops 2003 and 2004) were the source of the beans used in the experiments. Seven independent batches of flakes from each year were prepared for extracts and used for experiments. All extracts were tested for the presence of LOX 1, 2 and 3. Each experiment was replicated three times using each of the extracts. Heating, substrate and dissolved oxygen experiments were done for both years of beans (crop 2003 and 2004); pH experiment and LOX inhibitors experiments were done with beans from crop 2004.

Results and Discussion

Volatile Production and the Effect of Damage

Several studies have reported that LOX-null soybeans and oils produced off-flavor during storage [10, 11]. In the present study, the volatiles profile of dry LOX-null soybeans consistently contained oxidative flavor products, such as hexanal, hexenal, nonanal and 1-octen-3-ol, compounds associated with LOX activity (Table 1). These LOX triple-null soybeans were tested for the presence of residual LOX 1, 2 and 3 by the method of Hammond et al.

Table 1 Mean values ($n = 3$) of volatile compounds of LOX-null hand-harvested dry beans week 0, dry beans week 7, crushed beans week 0, and week 0 beans soaked in water for 2 h, (Relative peak area/10,000)

Compound	Week 0 dry beans	Week 7 dry beans	Week 0 crushed beans	Week 0 soaked beans
Hexanal	0.73^{c}	41.0^{b}	95.5^{a}	23.6^{b}
Hexenal	_	1.98^{c}	44.9^{a}	10.4^{b}
1-octen-3-ol	1.08^{c}	26.2^{b}	10.5^{b}	470.8^{a}
Pentyl furan	0.94^{c}	8.51^{b}	17.5^{ab}	25.4^{a}
Octanone	_	7.06^{a}	10.2^{a}	12.4^{a}
Octanal	_	5.54^{c}	62.2^{a}	19.8^{b}
Octenal	_	20.6^{a}	25.6^{a}	11.18^{a}
Nonanone	_	0.86^{c}	19.4^{a}	8.08^{b}
Nonanal	5.64^{c}	41.3^{b}	72.0^{a}	42.4^{b}
Nonenal	0.72^{b}	9.92^{a}	10.2^{a}	16.5^{a}
Decanone	_	8.05^{a}	12.4^{a}	10.2^{a}
tt-2-4-nonadienal	1.41^{b}	44.5^{a}	11.43^{b}	50.2^{a}
tt-2-4-decadienal	_	13.1^{b}	23.3^{a}	26.9^{a}

Values in the same line with the same superscript letters are not significantly different ($p < 0.05$)

[9]. Negative reactions were observed for all three lipoxygenases. Mechanical damage of soybeans (crushing) increased the amounts of hexanal, hexenal, pentyl furan, octanone, octenal, nonanone, nonanal, and *tt*-2-4-decadienal from LOX-null soybeans. Similarly, the amounts of volatiles from LOX-null beans were elevated when intact LOX-null soybeans were soaked in water for 2 h and then volatile compounds were analyzed (Table 1). Because it was clear that damage caused off-flavor development, some soybeans were harvested carefully by hand to limit damage, and their volatile profile was studied over a 2-month period. The hand-harvested soybeans produced only very small amounts of hexanal and nonanal at day zero but over time the amounts and variety of the volatiles increased as shown in Table 1.

Effect of Heat Treatment on the Volatiles Profile and Oxygen Uptake

Heat treatment followed by dissolved oxygen analysis was performed to determine the nature of volatile compounds formation. Heat treatment significantly decreased the amounts of hexanal, 1-octen-3-ol, pentylfuran, octanone, octenal and nonenal detected in LOX-null defatted flakes incubated with LOX-null soybean oil when compared to its unheated control (Table 2). These results suggested a possible enzymatic nature of volatile production in

Table 2 Reduction (%) in volatile compounds in heated flakes incubated with crude soybean oil compared to unheated control flakes incubated with crude soybean oil (Reduction (%) = 100% − $Wt_{\text{volatile compound in heated flakes}} \times 100\%/Wt_{\text{volatile compound in unheated flakes}}$, $n = 3$)

Volatile compounds	Reduction in volatile compounds (%)
Hexanal	46.1
1-Octen-3-ol	97.3
Pentylfuran	99.5
Octanone	95.3
Octenal	66.9
Nonanal	81.6
Decanal	99.9

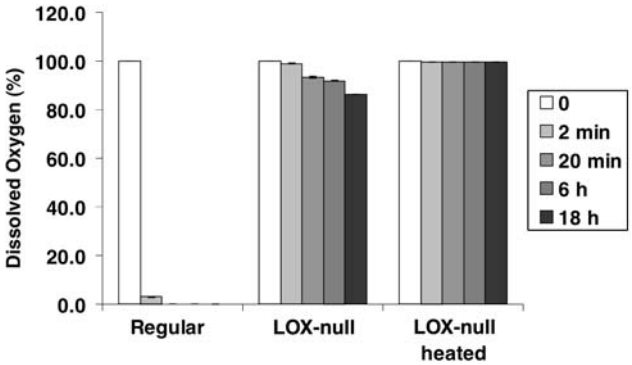

Fig. 1 Change in dissolved oxygen (%) when soybean oil is incubated with defatted soy flakes from conventional soybeans, LOX-null soybeans and, heated (60 °C) LOX-null soybeans

LOX-null beans and that heating for 10 min at 60 °C was sufficient to inactivate enzymes.

This hypothesis was supported by measurement of oxygen uptake. A mixture of refined (commercial) soybean oil and Century flakes consumed 99.9% of oxygen in 20 min (Fig. 1). A mixture of crude oil (neutral lipids, phospholipids, free fatty acids etc.) extracted from LOX-null soybeans and LOX-null defatted flakes consumed 6% of oxygen in 20 min and 13% of oxygen in 18 h (Fig. 1). Oxygen uptake in LOX-null flakes as well as in Century flakes was inactivated by heating for 10 min at 60 °C. Both mixtures of heat-treated oil and heat-treated flakes lost less than 0.5% of oxygen in 18 h (Fig. 1). Those results suggested that heating inactivated enzymes in regular and LOX-null soybeans. We were unable to show oxygen uptake by LOX-null flakes when commercial soybean oil was incubated with LOX-null flakes. This suggested that the LOX-like enzyme in LOX-null soybeans cannot use triacylglycerols as the substrate unlike the LOX in the Century flakes.

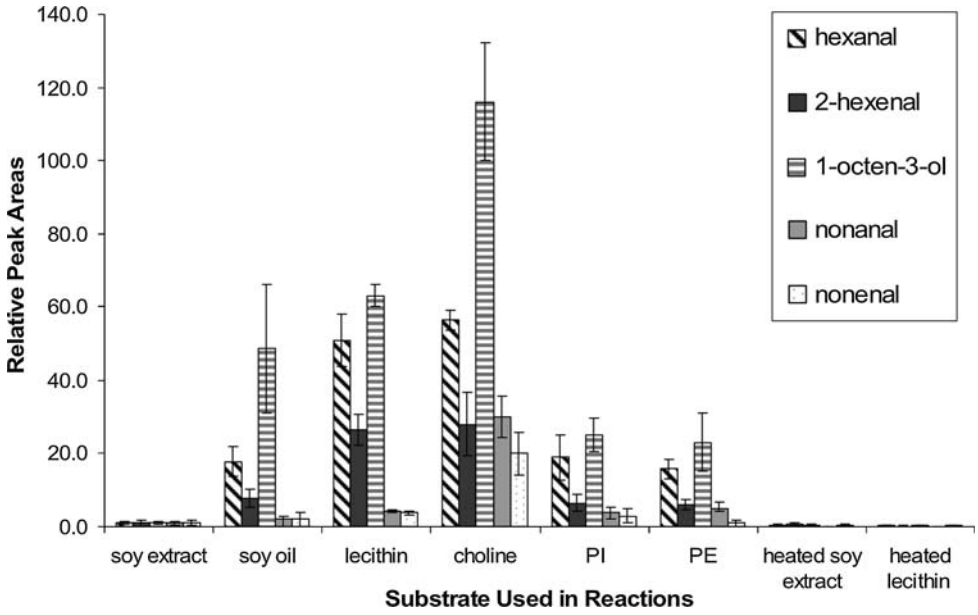

Fig. 2 Relative production of oxidative volatiles from defatted LOX-null soy flakes extract incubated with various substrates: extract of LOX-null flakes no added substrate, crude soybean oil (soy oil), soy lecithin, soy phosphatidylcholine (choline), soy phosphatidylinositol (PI), soy phosphatidylethanolamine (PE), and heated LOX-null extract with lecithin (heated lecithin). Background levels of volatiles in soy extract without added substrate was 1.0

Substrate Experiments

To determine the most efficient substrate for the putative LOX-like enzyme, LOX-null soy extracts were incubated with a variety of substrates including crude soybean oil, PE, PC, and PI., and soybean FFA. Incubating with soy lecithin resulted in higher volatiles production than incubating with soybean oil (Fig. 2). When FFAs were incubated with LOX-null soybean extract, the volatile production was not significantly different than that of crude soybean oil (data not shown). Crude soybean oil is unrefined and contains FFA.

Moreover, incubating soy phosphatidylcholine with LOX-null soy extract demonstrated the highest level of volatile production, but neither phosphatidyl-inositol nor -ethanolamine produced amounts of volatiles significantly different from the control (Fig. 2) when incubated with the extract. Volatile production was almost eliminated when soy extract was heated for 10 min at 60 °C and then incubated with soy lecithin or with crude soybean oil (Fig. 2).

Effect of LOX Inhibitors

Macrï et al. [12] have shown that LOX activity at the enzyme level may be inhibited by some antioxidants NDGA and propyl gallate. Production of hexanal, nonanal, nonenal and 1-octen-3-ol from defatted LOX-null soy flakes extract incubated with soy lecithin was significantly inhibited by propyl gallate (0.6 μM) and NDGA (200 μM) (Fig. 3). Propyl gallate and NDGA are potent antioxidants when used in unsaturated oils. In the current usage, these

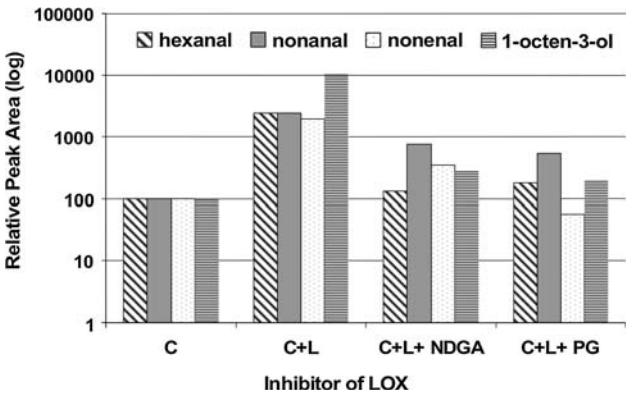

Fig. 3 Relative amounts (on a log scale) of volatile compounds produced when LOX-null extract is incubated with LOX inhibitors. LOX-null soy extract alone—C; LOX-null soy extract with soy lecithin—C + L; LOX-null soy extract with soy lecithin and 200 μM NDGA—C + L+NDGA: LOX-null soy extract with soy lecithin and 0.6 μM propyl gallate—C + L+PG

are specific inhibitors of LOX enzymes with propyl gallate being a competitive inhibitor while NDGA has been shown to be a non-competitive inhibitor of LOX activity.

Effect of pH on Volatiles Production

Production of volatile compounds from buffered liquid LOX-null soy extract incubated with soy lecithin was studied over the pH range from 3 to 10 with acetate buffer for pH 3–5.5, potassium phosphate buffer 5.5–8 and borate buffer for pH 8.0–10.0. The highest amounts of hexanal, 1-octen-3-ol and nonenal were obtained at pH 6.8 (Fig. 4). These results suggested that possible pH optimum for putative enzyme is near pH 6.8 region.

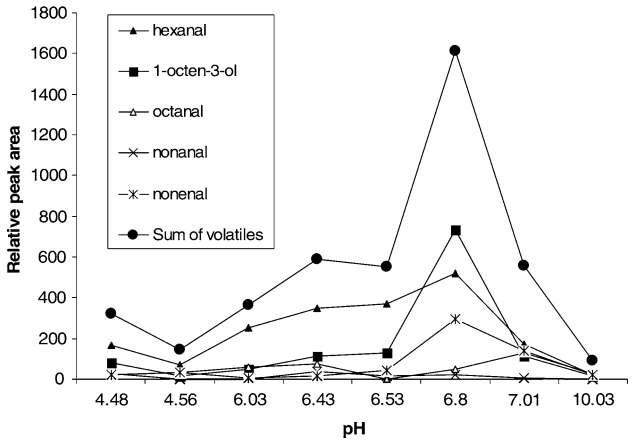

Fig. 4 Effect of pH on volatile compound production by LOX-null extract incubated with soy lecithin

LOXs are produced early in the germination of seeds [13]. The function of these LOXs is somewhat unclear, but the compounds that are produced may assist with development and/or protection of the seedlings. Of particular note is a membrane-bound LOX that is found within 11 days after placing a soybean under germination conditions [12, 14]. While immunologically similar to LOX-1, it appears to be membrane-bound and present early in germination. Free linoleic and linolenic acids, as well as soy phospholipids and triacylglycerols, can serve as substrates for soybean LOX 1, 2 and 3 [15–18].

In our labs and at NASA Johnson Space Center, (L. Wilson, personal communication) we have found that stored LOX triple-null beans develop volatiles that are consistent with a LOX-like enzyme. The level of hexenal in conventional beans is around 4.5 times greater than in triple-null beans. This is mitigated by the fact that upon one year of storage the level of hexenal in the triple-null beans increased up to fivefold. We were able to eliminate the activity of this LOX-like enzyme through heating and have determined crude pH and temperature optima. Interestingly, the substrate for this putative enzyme does not appear to be free polyenoic fatty acids but rather intact phospholipids. Thus, the cause of the off-flavors in LOX-null beans appears to have enzyme-like characteristics.

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