JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Identification of Linoleic Acid Hydroperoxides Generated by Soybean Seed Lipoxygenases 2 and 3

HIROTADA FUKUSHIGE,[†] CUNXI WANG,[†,‡] THOMAS D. SIMPSON,[§] HAROLD W. GARDNER,[§] AND DAVID F. HILDEBRAND*[,†]

Department of Agronomy, University of Kentucky, Lexington, Kentucky 40546, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

It has been known that lipoxygenase (LOX) isozymes exhibit differences in product formation, but most product information to date is for LOX 1 among soybean (*Glycine max*) LOX isozymes. In this study, LOXs 2 and 3 were purified and used to generate hydroperoxide (HPOD) products in an *in vitro* system using linoleic acid as a substrate in the presence of either air or O_2. The products were analyzed to determine their stereoisomeric characteristics. The control (no enzyme) showed only low levels of hydroperoxide production and no stereoisomeric specificity. LOX 2 shows high specificity in product formation, producing roughly 4 times more 13-HPOD than 9-HPOD, nearly all of which was 13-*S*(*Z*,*E*)-HPOD. LOX 3 produced more 9-HPOD than 13-HPOD at approximately a 2:1 ratio. No single stereoisomer was predominant among LOX 3 products. These results demonstrate that different isozymes of LOX have characteristic product profiles in *in vitro* reactions.

KEYWORDS: Lipoxygenase; linoleic acid hydroperoxide; autoxidation; soybean; *Glycine max*

INTRODUCTION

Lipoxygenases (LOXs) (EC 1.13.11.12) are a class of enzymes, widely distributed in both the plant and animal kingdoms, which oxidize unsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene system such as found in linoleic (18:2), linolenic, and arachidonic acids (*1*−*5*). Lipoxygenase produces hydroperoxides by the stereospecific abstraction of a hydrogen atom from position 3 of the penta-1,4-diene system, which may then disintegrate to give a proton (H^+) and an electron. Lipoxygenases contain a molecule of non-heme iron capable of capturing the electron of the hydrogen atom and producing an intermediate fatty acid radical, which reacts with oxygen in the triplet state to give a peroxyl radical. The enzyme-bound iron releases the captured electron, generating a peroxy anion, which, following protonation, produces the hydroperoxide. After a single electron shift, a 1-*cis*,3-*trans*-diene system is produced (*6*). These 1,3-dienes absorb strongly in the ultraviolet region, unlike 1,4-dienes; therefore, their production provides the basis for an assay of LOX activity (*7*).

In soybean [*Glycine max* (L.) Merr.] seeds, three distinct isoforms of LOXs have been described, based on differences in pH optima, substrate specificity, and their product formation (*7*). LOX type 1 (LOX 1) has a pH optimum of 9.0; LOX type 2 (LOX 2) has a pH optimum of pH 6.1; and LOX type 3 (LOX 3) has a pH optimum of pH 6.5. Arachidonic acid is the preferred substrate for LOX 2, although this is not a common constituent of plant fatty acids, whereas LOX 1 and LOX 3 are more active against 18:2. It was reported that the major product of LOX 1 activity with 18:2 is 13-hydroperoxy linoleic acid (HPOD); LOX 2 was said to form almost equal proportions of 9- and 13-HPODs and LOX 3 to produce approximately 65 and 35% 9- and 13-HPODs, respectively, although no determination as to the stereoisomeric forms of the products was performed (*7*). Christopher et al. (*8*) showed that the ratio of 13-HPOD/9-HPOD from the LOX 2 reaction ranged from 30:70 to 62:38 depending on the conditions employed. The stereochemistry of the LOX 1 products has been covered in some detail previously (*9*−*12*). On the other hand, beyond determining whether 9- or 13-HPODs were produced and their relative proportions, little work has been done on the products of LOXs 2 and 3 or further identification of the hydroperoxides and their stereospecificity. Maccarrone et al. (*13*) showed that LOX 2 produced 13-HPOD at about 28% of total HPODs from free 18:2 and the ratio of 13*S*-/13*R*-HPOD as 55:45 but did not show the stereospecificity of 9-HPOD.

Once LOX has produced fatty acid hydroperoxides, a series of enzymic reactions that further metabolize the hydroperoxides takes place, giving rise to a variety of oxygenated products called oxylipins (*1*, *3*, *14*−*16*). There are two major oxylipin pathways operating ubiquitously in plant leaf tissues. One is the allene oxide synthase pathway resulting in jasmonic acid and its derivatives. These compounds are well-documented to be signal compounds in stress responses such as wounding and pest attack

* To whom correspondence should be addressed. Telephone: 859-257-5020 ext 80760. E-mail: dhild@uky.edu.
[†] University of Kentucky.
[‡] Present address: Pioneer Hi-Bred International, A DuPont Company, Johnston, IA 50131.
[§] U.S. Department of Agriculture.

10.1021/jf047958o CCC: $30.25
Published on Web 06/16/2005

(*17*, *18*). The other pathway is the hydroperoxide lyase pathway producing volatile aldehyde and alcohol compounds such as (2*E*)-hexenal and (3*Z*)-hexenol. These volatiles are associated with "fresh" green odors of cut leaves and also important components of food flavors (*19*, *20*).

Because many of the oxylipins have been shown to be biologically active in both plants and animals, an understanding of the variety and specificity of the formation of fatty acid hydroperoxides by given LOXs on defined substrates will further enhance our understanding of the subsequent production of these important compounds. In this study, we examined the products formed using 18:2 as the substrate by LOXs 2 and 3. To this end, LOX isozymes were purified to near homogeneity, so as to remove the enzymes metabolizing hydroperoxides and reacted with 18:2 as the substrate in an *in vitro* assay. The products were extracted, derivatized, and analyzed on a series of chiral HPLC columns.

MATERIALS AND METHODS

Plant Material. The soybean [*Glycine max* (L.) Merr.] lipoxygenase (LOX) mutant lines −L2L3, −L1L3, and −L3 backcrossed to Century were provided by Niels Nielsen, Purdue University, West Lafayette, IN (*21*). −L2L3 line is a null for LOX 2 and 3 (but has the normal wild-type level of LOX 1); −L1L3 line only contains LOX 2; and −L3 lacks LOX 3. Century seeds contain LOXs 1, 2, and 3.

Purification of LOX 2 and 3 from Dry Soybean Seeds. To facilitate purification, two mutants of soybean seeds, −L1L3 and −L1L2, were used for preparation of LOXs 2 and 3. Approximately 2 g of dry soybean seeds were ground to a fine meal using an electric coffee bean grinder, and the powder was extracted in a prechilled mortar and pestle on ice with 20 mL of deionized water at 4 °C. The resultant slurry was passed through two layers of Miracloth (EMD Biosciences, San Diego, CA), and $CaCl_2$ was added to a final concentration of 43 mM followed by incubation on ice for 30 min to precipitate the storage proteins. The solution was centrifuged at 12000*g* at 4 °C for 10 min, and the supernatant was filtrated through 2 layers of cheesecloth and taken to a final concentration of 25% ammonium sulfate at 4 °C. After incubation on ice for 60 min to precipitate proteins, centrifugation was performed as before for 15 min. The supernatant was carefully removed, and the ammonium sulfate concentration increased to 60%. A further 60 min incubation on ice was performed before centrifugation. The supernatant was discarded, and traces of liquid were carefully removed from the tube. The remaining pellet was resuspended in 5 mL of ice-cold deionized water and then desalted on a 23 × 2.5 cm Sephadex G50 column (column bed volume of 65 cm^3) at 4 °C using buffer A (10 mM EDTA, 0.1% Tween 20, and 10 mM sodium phosphate at pH 6.8) as the desalting buffer. A_{280} absorbing fractions of 4 mL were collected, and LOX activity was measured spectrophotometrically as previously described (*22*). Fractions showing LOX activity were pooled, and the LOX isozymes were separated using a Pharmacia FPLC system (Amersham Biosciences, Piscataway, NJ). The LOX fractions were passed through a 5 μm Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI) to remove particulate matter and loaded onto a MonoQ HR 5/5 column using buffer A. The column was washed with buffer A until UV-absorbing material no longer eluted from the column. Bound proteins were eluted from the column using a linear gradient of buffer B (0.5 M NaCl, 10 mM EDTA, 0.1% (v/v) Tween 20, and 10 mM sodium phosphate at pH 6.8) at a flow rate of 1 mL min^{-1}. Fractions of 0.5 mL were collected and individually assayed for LOX activity. It was previously determined which fractions represented the individual LOXs present (*23*). To check the level of enzyme purification, 10 μg of protein was loaded onto a 10% SDS−PAGE gel, electrophoresed, and then stained with Coomassie blue. No other proteins were detectable at this point other than single bands at approximately 100 kD. Western analysis of gels using a polyclonal soybean LOX antibody further identified these proteins as LOXs.

LOX Substrate Preparation and Reaction Conditions. A 10% (v/v) linoleic acid (18:2) substrate stock in 95% ethanol was prepared. The substrate stock was flushed with argon prior to storage at −20 °C. For enzyme assays, 0.1 mL of the fatty acid stock was mixed with 10 mL of 10 mM KOH and then 0.1 mL of this solution was mixed with 100 mL of 0.1 M potassium phosphate buffer at pH 6.6. For the assay, 1 mL of the appropriate buffer was mixed with 0.1 mL of the buffered substrate and between ~0.75 and 1 mL of the purified enzyme was added. The reaction was performed in a 16 × 125 mm glass test tube at 20 °C in a water bath. The reaction was continuously stirred using a Teflon-coated microstirring bar and either air or O_2 gas supplied via Teflon tubing for aeration at a sufficient rate but low enough to prevent foaming. After incubation for 30 min, the LOX products were isolated and purified for further analysis.

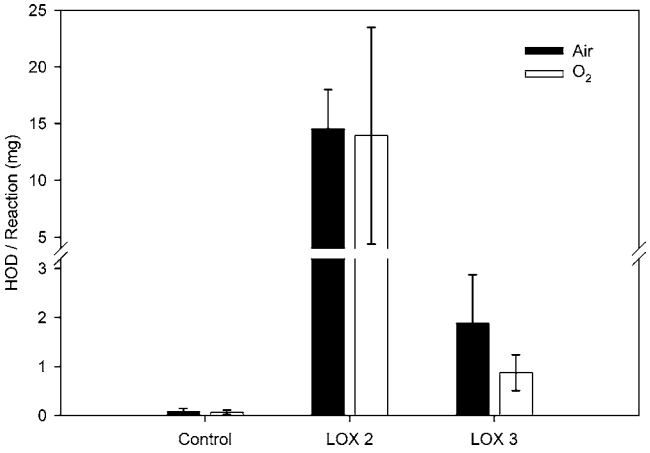

Figure 1. Comparison of the total HPOD production with purified LOX 2 and LOX 3 in the presence of either air or O_2. Control reactions contain no LOXs. HPODs were reduced to HODs prior to HPLC analysis. Each value was the average and standard error of two replications.

HPOD Product Extraction and Derivatization. An equal volume of HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA) was added to each reaction and adjusted to pH 4 using 1 M oxalic acid and 2 volumes of chloroform. The sample was vigorously vortexed, and the phases were allowed to separate. The chloroform phase was carefully removed, and the methanol-aqueous phase was re-extracted twice with an equal volume of chloroform. The chloroform fractions were pooled (total volume of ca. 8 mL) and evaporated using GC-grade nitrogen gas to a final volume of approximately 1 mL. To this was added 1 mL of a 38 mM triphenylphosphine solution in ether to reduce HPODs to hydroxy linoleic acids (HODs). After mixing, the solution was placed in a 4 °C refrigerator for several hours and the solvent was allowed to evaporate slowly. The remaining solution containing derivatized linoleic hydroperoxides was mixed with 1 mL of chloroform and stored at −70 °C until analysis. Control reactions contained all of the above components except that no enzyme was added. Incubation time and product extraction was exactly the same as for the enzymatic reactions.

HPLC Analysis and Identification. Samples were analyzed on a Spectra-Physics HPLC system consisting of an Iso Chrom LC Pump, a SP8490 UV detector, and a SP4270 Integrator (Spectra-Physics, Mountain View, CA) using a Bakerbond Ultrasphere chiral column (DNBPG chiral stationary phase, ionic, 4.6 × 250 mm, 5 μm particle size) (Mallinckrodt Baker, Phillipsburg, NJ). The determination used an isocratic hexane/2-propanol (99.7:0.3, v/v) solvent with flow of 0.75 mL min^{-1} and a UV detector set at 235 nm.

RESULTS

HPODs were produced in the control reactions when either air or O_2 were supplied to the reactions but were probably formed by autoxidation of the substrate and not enzymatically. Control reactions using air produced only about 4% of the HPOD seen when LOX 3 enzyme was present and around 0.5% of the levels of HPOD generated with LOX 2 present (**Figure 1**). When O_2 gas was supplied to the reaction, the results were similar, although the LOX 3 reaction produced around 50% less

Table 1. Stereoisomeric Forms of HPOD Produced during Control Reactions and in the Presence of LOX Isozymes Using Linoleic Acid as a Substrate in the Presence of Either Air or O_2[a]

		13-HOD				9-HOD			
isozyme	source of oxygen	*S*(*Z*,*E*)	*R*(*Z*,*E*)	*S*(*E*,*E*)	*R*(*E*,*E*)	*S*(*Z*,*E*)	*R*(*Z*,*E*)	*S*(*E*,*E*)	*R*(*E*,*E*)
LOX 2	air	75.1 ± 0.6	2.3 ± 0.2	2.5 ± 0.1	1.5 ± 0.1	12.7 ± 0.1	2.6 ± 0.0	1.8 ± 0.2	1.6 ± 0.2
LOX 2	O_2	76.7 ± 1.7	2.4 ± 0.3	2.5 ± 0.1	1.4 ± 0.2	11.5 ± 0.3	2.4 ± 0.2	1.8 ± 0.2	1.7 ± 0.2
LOX 3	air	6.8 ± 1.0	5.9 ± 0.4	13.6 ± 0.4	10.7 ± 0.3	21.8 ± 1.4	21.8 ± 1.3	9.8 ± 0.3	9.7 ± 0.4
LOX 3	O_2	12.5 ± 2.1	5.5 ± 0.3	11.0 ± 0.6	10.2 ± 0.4	23.5 ± 0.1	18.5 ± 0.0	9.3 ± 0.6	9.7 ± 0.3

[a] The value is the percentage of each stereoisomeric form to the total HOD produced (average and standard error of two replications).

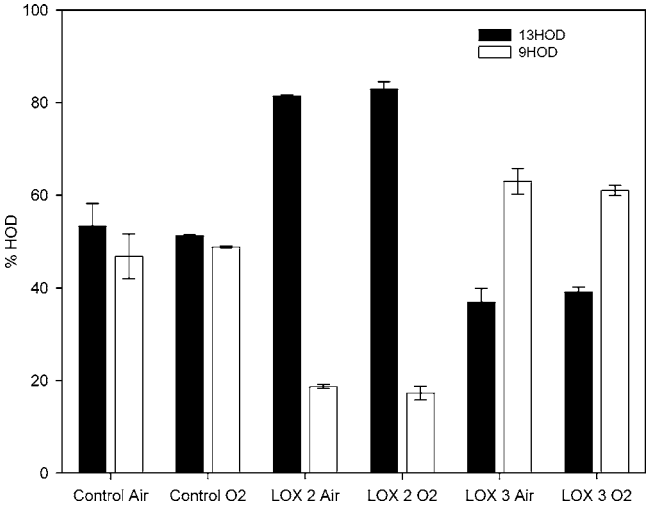

Figure 2. Comparison of the percentage of 9-HPOD and 13-HPOD production with LOX 2 and LOX 3 in the presence of either air or O_2. HPODs were reduced to HODs prior to HPLC analysis. The value was expressed as a percentage of the total HPOD production and the average and standard error of two replications.

product when O_2 gas was supplied instead of air. The control reactions also produced approximately equal proportions of 9- and 13-hydroperoxide with both air and O_2, whereas the regiospecificity of LOX 2 and LOX 3 appeared to favor the production of one HPOD type over the other (**Figure 2**). LOX 2 generated more 13-HPOD at 82% of total HPOD compared to 18% of 9-HPOD (13-HPOD/9-HPOD was 4.55:1). LOX 3 preferentially produced more 9-HPOD than 13-HPOD with 62 and 38% of total HPOD, respectively (13-HPOD/9-HPOD was 1:1.6).

In the LOX 2 reactions supplied with either air or O_2, two stereospecific products predominated: 13-*S*(*Z*,*E*)-HPOD was the major product, comprising about 75% of total HPOD, and the next largest product formed was 9-*S*(*Z*,*E*)-HPOD, which was around 12% of total HPOD generated (**Table 1**). The other stereoisomers made up the remaining HPOD total in roughly equal proportions. Results from LOX 3 air or O_2-aerated reactions were also very similar in their results. Here, the 9-HPODs were preferentially formed over 13-HPODs. 9-*S*(*Z*,*E*)-HPOD and 9-*R*(*Z*,*E*)-HPOD each comprised around 20% of total HPODs formed. The remaining 9- and 13-HPOD geometric isomers (*Z*,*E* and *E*,*E*) each accounted for between 6 and 14% of total HPOD produced. The HPOD stereoisomers produced in air or O_2 control reactions were racemic, but the variation among replications was high because of the small size of products produced (data not shown).

DISCUSSION

An autoxidative process, presumably the main factor in the control reactions (no added enzyme), produced approximately equal amounts of 9- and 13-HPODs from 18:2 in our *in vitro* system, although the absolute amount of HPODs produced was significantly less than the amounts generated when enzyme was added (**Figure 1**). When purified enzymes were added to the reactions, HPOD production was much greater than in the controls and the stereoisomeric HPODs produced showed a different profile to the autoxidation-generated HPODs. The LOX 2 isozyme generated more 13-HPOD than 9-HPOD with around 80% of total HPOD as the former, regardless of whether air or O_2 was used. The major HPOD produced was 13-*S*(*Z*,*E*)-HPOD, being about 75% of the total HPOD produced. The purified LOX 2 was much more active against 18:2 than LOX 3 and generated larger amounts of product. LOX 3 reactions preferentially produced more 9-HPOD than 13-HPOD, the opposite of what was seen with LOX 2, although the differences in amounts were not as clear as with LOX 2, showing around 40% 13-HPOD and 60% 9-HPOD production. Unlike the LOX 2 reactions, no predominating stereoisomeric HPOD was produced, with all stereoisomers being represented at comparable levels. These results show that the LOX 2 enzyme has a much greater specificity in its product formation than does LOX 3, at least in this *in vitro* system.

Previous attempts to determine the specificity of product formation by different LOX isozymes have revealed significant effects of the conditions employed (*7*, *8*, *24*). In our present experiments, LOX 2 produced more 13-HPOD than 9-HPOD (80:20) from 18:2 (3.21 μM). Christopher et al. (*8*), who noted various ratios of 13- and 9-HPOD in LOX 2 reactions, used 1.8 mM 18:2 as a substrate. Maccarrone et al. (*13*), whose substrate concentration was 300 μM, reported more 9-HPOD production than 13-HPOD from free 18:2 while showing that more 13-HPOD is produced from a membrane lipid pool as a substrate. Haslbeck and Grosch (*25*) argued that at concentrations of 18:2 above 100 μM, the product specificity of LOX 2 from 18:2 is more similar to the results from autoxidation because of the reaction between excess 18:2 and released hydroperoxyl radicals. In their experiments, LOX 2 produced an almost equal amount of 13- and 9-HPOD from 1.3 mM 18:2 alone. However, when crocin, a digentabiose ester of crocetin, was included in the reactions as a co-oxidant, more 13-HPOD was produced. They attributed this shift to the elimination of released hydroperoxyl radicals by crocin, resulting in the reduced autoxidative formation of hydroperoxide isomers.

On the whole, our results for LOX 3 agreed fairly well with those of Axelrod et al. (*7*). Gardner et al. (*26*) stated that LOX 2 behaved almost like an "acidic pH LOX 1" in that, at its pH optimum, it produced one predominating product, whereas the LOX 3 products were almost racemic. This difference between LOX 2 and 3 is also seen at the level of the different stereoisomers of product produced, in that LOX 2 favors the production of one isomeric form. LOX 3, however, again produces almost racemic mixes of the possible isomers, showing

that there is a clear difference in product specificity between the two enzymes.

Use of purified enzymes in an *in vitro* system supplied with only one substrate type while other enzymes found in the LOX pathway are not present has allowed us to determine the specificity of the isozymes and demonstrate clear differences between LOXs 2 and 3. Caution must be taken in extrapolating these results back to what may actually be occurring *in planta*, where other substrates, isomerases, and LOX pathway enzymes might be present in an entirely different physicochemical environment.

ABBREVIATIONS USED

18:2, linoleic acid; HOD, hydroxy linoleic acid; HPOD, hydroperoxy linoleic acid; LOX, lipoxygenase.

LITERATURE CITED

(1) Feussner, I.; Wasternack, C. The lipoxygenase pathway. *Annu. Rev. Plant Biol.* **2002**, *53*, 275−297.

(2) Hildebrand, D. F.; Hamilton-Kemp, T. R.; Legg, C. S.; Bookjans, G. Plant lipoxygenases: Occurrence, properties, and possible functions. *Curr. Top. Plant Biochem. Physiol.* **1988**, *7*, 201−219.

(3) Porta, H.; Rocha-Sosa, M. Plant lipoxygenases. Physiological and molecular features. *Plant Physiol.* **2002**, *130*, 15−21.

(4) Siedow, J. N. Plant lipoxygenase: Structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145−188.

(5) Shibata, D.; Axelrod, B. Plant lipoxygenases. *J. Lipid Mediat. Cell Signal.* **1995**, *12*, 213−228.

(6) Taylor, G. W.; Morris, H. R. Lipoxygenase pathways. *Brit. Med. Bull.* **1983**, *39*, 219−222.

(7) Axelrod, B.; Cheesbrough, T. M.; Laakso, S. Lipoxygenase from soybeans. *Methods Enzymol.* **1981**, *71*, 441−451.

(8) Christopher, J. P.; Pistorius, E. K.; Regnier, F. E.; Axelrod, B. Factors influencing the positional specificity of soybean lipoxygenase. *Biochim. Biophys. Acta* **1972**, *289*, 82−87.

(9) Andre, J. C.; Funk, M. O. Determination of stereochemistry in the fatty acid hydroperoxide products of lipoxygenase catalysis. *Anal. Biochem.* **1986**, *158*, 316−321.

(10) Gardner, H. W. Soybean lipoxygenase-1 enzymically forms both (9*S*)- and (13*S*)-hydroperoxides from linoleic acid by a pH-dependent mechanism. *Biochim. Biophys. Acta* **1989**, *1001*, 274−281.

(11) Hamberg, M. Steric analysis of hydroperoxides formed by lipoxygenase oxygenation of linoleic acid. *Anal. Biochem.* **1971**, *43*, 515−526.

(12) van Os, C. P. A.; Vente, M.; Vliegenthart, J. F. G. A NMR shift method for determination of the enantiomeric composition of hydroperoxides formed by lipoxygenase. *Biochim. Biophys. Acta* **1979**, *574*, 103−111.

(13) Maccarrone, M.; van Aarle, P. G. M.; Veldink, G. A.; Vliegenthart, J. F. G. *In vitro* oxygenation of soybean biomembranes by lipoxygenase-2. *Biochim. Biophys. Acta* **1994**, *1190*, 164−169.

(14) Blée, E. Impact of phyto-oxylipins in plant defense. *Trends Plant Sci.* **2002**, *7*, 315−322.

(15) Howe, G. A.; Schilmiller, A. L. Oxylipin metabolism in response to stress. *Curr. Opin. Plant Biol.* **2002**, *5*, 230−236.

(16) Schaller, F. Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. *J. Exp. Bot.* **2001**, *52*, 11−23.

(17) Turner, J. G.; Ellis, C.; Devoto, A. The jasmonate signal pathway. *Plant Cell* **2002**, *14*, S153−S164.

(18) de Bruxelles, G. L.; Roberts, M. R. Signals regulating multiple responses to wounding and herbivores. *Crit. Rev. Plant Sci.* **2001**, *20*, 487−521.

(19) Hatanaka, A. The biogeneration of green odour by green leaves. *Phytochemistry* **1993**, *34*, 1201−1218.

(20) Whitehead, I. M.; Muller, B. L.; Dean, C. Industrial use of soybean lipoxygenase for the production of natural green note flavor compounds. *Cereal Foods World* **1995**, *40*, 193−194, 196−197.

(21) Davies, C. S.; Nielsen, N. C. Registration of soybean germplasm that lacks lipoxygenase isozymes. *Crop Sci.* **1987**, *27*, 370−371.

(22) Hildebrand, D. F.; Versluys, R. T.; Collins, G. B. Changes in lipoxygenase isozyme levels during soybean embryo development. *Plant Sci.* **1991**, *75*, 1−8.

(23) Wang, C.; Croft, K. P. C.; Hildebrand, D. F. α-Naphthaleneacetic acid induces the expression of seedling lipoxygenases in cultured immature zygotic soybean embryo cotyledons. *Plant Cell Rep.* **2001**, *20*, 85−91.

(24) Christopher, J.; Axelrod, B. On the different positional specificities of peroxidation of linoleate shown by two isozymes of soybean lipoxygenase. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 731−736.

(25) Haslbeck, F.; Grosch, W. HPLC analysis of all positional isomers of the monohydroperoxides formed by soybean lipoxygenases during oxidation of linoleic acid. *J. Food Biochem.* **1985**, *9*, 1−14.

(26) Gardner, H. W.; Takamura, H.; Hildebrand, D. F.; Croft, K. P. C.; Simpson, T. D.; Salch, Y. P. Oxylipin pathway in soybeans and its physiological significance. In *Lipoxygenase and Lipoxygenase Pathway Enzymes*; G. Piazza, Ed.; AOCS Press: Champaign, IL, 1996; pp 162−175.

Received for review December 3, 2004. Revised manuscript received April 14, 2005. Accepted April 19, 2005.

JF047958O