Contribution of Hydroperoxide Lyase Activity to *n*-Hexanal Formation in Soybean

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A hydroperoxide (HPO) lyase which catalyzes the specific cleavage of 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-L-*c*,*t*-HPO) to form *n*-hexanal was found in the homogenates of the soybeans such as *Glycine max* var. Suzuyutaka (normal type) and lipoxygenase (L) deficient mutant seeds (L-1 null, L-2 null, L-3 null). A carbonyl compound which was formed from 13-L-*c*,*t*-HPO and 13-DL-*c*,*t*-HPO by the enzyme was *n*-hexanal. The enzyme was specific to the L isomer of 13-*c*,*t*-HPO. 9-D-*t*,*c*-HPO was not available for the enzyme reaction. K_m and V_{max} values of the enzyme for 13-L-*c*,*t*-HPO were apparently similar among all the seeds. Apparent V_{max} value of the homogenate from L-2 null seed for linoleic acid was considerably lower ($^1/_3$–$^1/_4$) than those in the other seeds. It is concluded that a rate-determining step of *n*-hexanal formation from linoleic acid in L-2 null seed is a step of 13-L-*c*,*t*-HPO formation by lipoxygenase.

INTRODUCTION

Characteristic grassy bean and green flavors build a barrier for wide utilization of soy proteins as food ingredients. Major contributors to these flavors are the volatile carbonyl compounds which are enzymatically derived from hydroperoxides of unsaturated fatty acids (Rackis et al., 1979). Soybean contains three kinds of lipoxygenase isozymes (lipoxygenase-1 (L-1), L-2, L-3) which exhibit different kinetic behaviors (Galliard and Chan, 1980; Axelrod et al., 1981). In the previous paper, we described the mechanism of the formation of *n*-hexanal, one of the major elements of soybean flavors, by using a wild type (normal seed) and lipoxygenase deficient mutants of soybeans (L-1 null, L-2 null, L-3 null, and L-1, -3 null), and that L-2 isozyme is responsible for *n*-hexanal formation from free linoleic acid as the substrate (Matoba et al., 1985). However, the pathway of *n*-hexanal formation from the hydroperoxides produced by lipoxygenase has not been clearly understood.

The presence of hydroperoxide lyase which cleaves fatty acid hydroperoxides has been established in cucumber fruits (Galliard and Philips, 1976; Galliard et al., 1976a,b), watermelon seedlings (Vick and Zimmerman, 1976), tomato fruits (Galliard and Matthew, 1977), pear fruits (Kim and Grosch, 1981), tea leaves chloroplast (Hatanaka et al., 1982a), and phaseolus leaves (Matthew and Galliard, 1978; Hatanaka et al., 1982b). However, no information about this enzyme has been shown in soybean seeds.

In the present paper, we have first shown the presence of hydroperoxide lyase in soybean, the enzymatic properties as the substrate specificity, kinetic parameters (K_m and V_{max}) and optimum pH, and a mechanism of *n*-hexanal formation in soybean.

MATERIALS AND METHODS

Soybean Cultivars. Soybeans were grown at the Iwate University Experimental Farm located in Iwate Morioka, Japan, in 1984. Normal seed (wild type) was *Glycine max* var. Suzuyutaka. L-1 deficient mutant was a line from the cross between P.I. 408251 (L-1 null type) and Suzuyutaka. L-2 deficient mutant was a line from the cross between P.I. 86023 (L-2 null type) and Suzuyutaka. L-3 deficient mutant was a line from the cross between Tohoku No. 74 (L-3 null type) and Suzuyutaka.

Reagents. (2,4-Dinitrophenyl)hydrazine, Tween 20, Tween 80, linoleic acid (>99%) and aldehydes were purchased from Nakarai Chemicals Ltd., Kyoto. Lipoxidase (type I) from soybean (containing lipoxygenase-1) was obtained from Sigma, St. Louis. (2,4-Dinitrophenyl)-hydrazones of aldehydes were synthesized by the procedure of Shriner et al. (1956). 13-DL-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-DL-*c*,*t*-HPO) and 13-DL-hydroperoxy-*trans*-9,*trans*-11-octadecadienoic acid (13-DL-*t*,*t*-HPO) were prepared from autoxidation products of linoleic acid, and 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-L-*c*,*t*-HPO) and 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9-D-*t*,*c*-HPO) were prepared from the reaction product of linoleic acid by commercial soybean lipoxygenase-1 according to the procedure of Egmond et al. (1976). All the hydroperoxides were purified with a high performance liquid chromatography (HPLC) on normal-phase column (YMC-Pack Sil). The purities of the hydroperoxides were more than 95%.

Preparation of Soybean Extracts. Soybean seeds (3–4 grains, about 0.5 g) were soaked in water at 4 °C overnight. After removal of seed coat, the soaked seeds were homogenized in 10 mL of cold water with a glass homogenizer (Potter-Elvejhem type) under cooling in an ice bath. The resulting homogenate was used for determining enzymatic parameters.

Determination of *n*-Hexanal. *n*-Hexanal was determined as the (2,4-dinitrophenyl)hydrazine derivative with HPLC as described in the previous paper (Matoba et al., 1985).

Measurement of Hydroperoxide Lyase Activity. The activity was measured with linoleic acid and its hydroperoxides (13-DL-*c*,*t*-HPO, 13-DL-*t*,*t*-HPO, 13-L-*c*,*t*-HPO, and 9-D-*t*,*c*-HPO) as the substrate according to the method of Grossman and Zakut (1979) and Kim and Grosch (1981) with some modifications. The reaction was carried out at 25 °C for 5 min in 0.1 M phosphate buffer (pH 7.0) containing different concentrations of the substrate (20–250 μM) and 0.004% Tween 20 or Tween 80. Tween 20 and Tween 80 were used for linoleic acid and hydroperoxides, respectively. The activity was determined by measuring formation of *n*-hexanal. Kinetic parameters, K_m and V_{max}, were calculated from Lineweaver–Burk plots.

Protein Content. The protein content of soybean extracts was determined by the procedure of Lowry et al.

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0021-8561/85/1433-0856$01.50/0

Table I. Relative Activity of *n*-Hexanal Formation from Various Hydroperoxides of Linoleic Acids[a]

substrate	relative activity, %	
	n-hexanal	*cis*-2-nonenal
	Suzuyutaka	
13-L-*c*,*t*-HPO	100	trace
13-DL-*c*,*t*-HPO	57	trace
13-DL-*t*,*t*-HPO	trace	trace
9-D-*t*,*c*-HPO	trace	trace
	L-1 null	
13-L-*c*,*t*-HPO	100	trace
13-DL-*c*,*t*-HPO	59	trace
13-DL-*t*,*t*-HPO	trace	trace
9-D-*t*,*c*-HPO	trace	trace
	L-2 null	
13-L-*c*,*t*-HPO	100	trace
13-DL-*c*,*t*-HPO	54	trace
13-DL-*t*,*t*-HPO	trace	trace
9-D-*t*,*c*-HPO	trace	trace
	L-3 null	
13-L-*c*,*t*-HPO	100	trace
13-DL-*c*,*t*-HPO	57	trace
13-DL-*t*,*t*-HPO	trace	trace
9-D-*t*,*c*-HPO	trace	trace

[a] 13-L-*c*,*t*-HPO, 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 13-DL-*c*,*t*-HPO, 13-DL-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; 13-DL-*t*,*t*-HPO, 13-DL-hydroperoxy-*trans*-9,*trans*-11-octadecadienoic acid; 9-D-*t*,*c*-HPO, 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid.

(1951) with bovine serum albumin as the standard.

RESULTS

Linoleic Acid Hydroperoxide Lyase Activity in the Homogenates of Various Soybeans. The homogenates of normal type (Suzuyutaka) and L-1, L-2, and L-3 null mutant seeds were allowed to react with 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic (13-L-*c*,*t*-HPO), 13-DL-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic (13-DL-*c*,*t*-HPO), 13-DL-hydroperoxy-*trans*-9,*trans*-11-octadecadienoic (13-DL-*t*,*t*-HPO), and 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acids (9-D-*t*,*c*-HPO) at 25 °C. Table I shows relative activities of cleavage of the hydroperoxides. In all the seeds, carbonyl compounds having more than five carbons (*n*-pentanal) were significantly detected in the reaction mixtures only when 13-L-*c*,*t*-HPO and 13-DL-*c*,*t*-HPO were used as the substrate, and the main product was *n*-hexanal. Figure 1 shows a typical profile of separation with HPLC when the homogenate of Suzuyutaka was allowed to react with 13-L-*c*,*t*-HPO or 9-D-*t*,*c*-HPO. The profile was similar to those obtained with the other seeds. The ratio of *n*-hexanal formation from 13-L-*c*,*t*-HPO and 13-DL-*c*,*t*-HPO was about 2:1. The activity was lost after a heat treatment of the homogenates at 100 °C for 5 min. Trace amounts of carbonyl compounds were detected in the reaction mixture containing 9-D-*t*,*c*-HPO as the substrate. From these results, it is likely that an enzyme (hydroperoxide lyase) which catalyzes the cleavage of 13-hydroperoxide of linoleic acid is present in soybean and that the substrate specificity of the enzyme is restricted for 13-L-*c*,*t*-HPO. In all the seeds, the optimum pH of the activity was 6–7, similar to that of soy milk.

Kinetic Parameters of *n*-Hexanal Formation from Linoleic Acid and 13-L-*c*,*t*-HPO. The homogenates of the normal type seed (Suzuyutaka) and L-1, L-2, and L-3 null mutant seeds were allowed to react with various concentrations of linoleic acid and 13-L-*c*,*t*-HPO. K_m and V_{max} values for *n*-hexanal formation were determined as shown in Table II. The apparent K_m (40–60 μM) and V_{max} (14–20 μmol/mg/min) values for 13-L-*c*,*t*-HPO were similar among all the seeds. The apparent K_m value of the homogenate enzyme system for *n*-hexanal formation from linoleic acid was 30–40 μM in Suzuyutaka, L-1, and L-2 null seeds, and 50 μM in L-2 null seed, showing that the value in L-2 null seed is slightly higher than those in the other seeds. The apparent V_{max} values from Suzuyutaka, L-1, and L-3 null seeds for linoleic acid which were obtained with the lyase reaction by coupling with lipoxygenase reaction were 10–20 μmol/mg/min which were very similar to those obtained for 13-L-*c*,*t*-HPO. On the other hand, the apparent V_{max} value for linoleic acid in L-2 null seed was considerably lower (4.5 μmol/mg/min) than those in the other seeds.

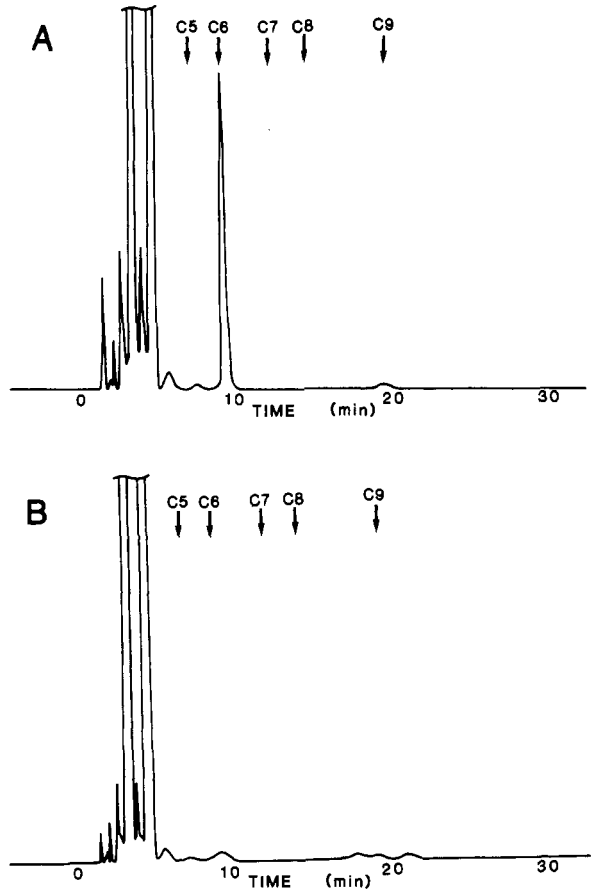

Figure 1. Separation of carbonyl compounds as their (2,4-dinitrophenyl)hydrazine derivatives after reaction with 9- and 13-hydroperoxides by high performance liquid chromatography. 13-L-*c*,*t*-HPO (A) or 9-D-*t*,*c*-HPO (B) was allowed to react with the homogenate of Suzuyutaka at 25 °C and for 20 min. The products were used for the analysis of carbonyl compounds as described under Methods: C_5, *n*-pentanal; C_6, *n*-hexanal; C_7, *n*-heptanal; C_8, *n*-octanal; C_9, *n*-nonanal.

Table II. Kinetic Parameters of *n*-Hexanal Formation from Linoleic Acid and 13-L-*c*,*t*-HPO[a]

	K_m, μM		V_{max}, μmol/mg/min	
	LA	HPO	LA	HPO
Suzuyutaka	38	44	11	14
L-1 null	40	55	14	19
L-2 null	59	59	4.5	16
L-3 null	31	39	17	22

[a] LA, linoleic acid; HPO, 13-L-*c*,*t*-HPO.

DISCUSSION

In the previous paper, we demonstrated that L-2 isozyme was responsible for *n*-hexanal formation in the soybean homogenates under the usual conditions (pH 6–7) and its

substrate was free linoleic acid (Matoba et al., 1985). As elucidated in the present study, *n*-hexanal formation was not caused by nonenzymatic reaction, but enzymatically generated by hydroperoxide lyase. Galliard and Chan (1980) and Axelrod et al. (1981) imply that 9- and 13-hydroperoxides are produced from linoleic acid by the enzyme in soy milk. However, we have shown that only *n*-hexanal was most abundantly detected among carbonyl compounds having more than five carbons (*n*-pentanal) in the soybean homogenates (Matoba et al., 1985). This may be explained by the evidence that only 13-hydroperoxide lyase is present in soybean. There have been various types of hydroperoxide lyases in plants. The enzyme is specific for 13-hydroperoxide in tomato fruits (Galliard and Matthew, 1977) and tea chloroplast (Hatanaka et al., 1981), specific for 9-hydroperoxide in pear fruits (Kim and Grosch, 1982), and specific for both 9- and 13-hydroperoxides in cucumber fruits (Galliard et al., 1976). The soybean lyase did not act on 13-hydroperoxide of methyl linoleate (data not shown). This strongly suggests that *n*-hexanal is enzymatically generated through 13-hydroperoxide from free linoleic acid but not from bound linoleic acid such as triacylglycerols and phospholipids.

Under the usual conditions (pH 6–7), the level of *n*-hexanal formation in L-2 null seed was considerably lower than those in the other seeds, though the lyase activity occurred in the same level as those in all the seeds.

From several lines of the evidence as described above, it is postulated that L-2 isozyme predominantly reacts with linoleic acid to produce 13-L-*c*,*t*-HPO under the usual conditions (pH 6–7) and that 13-L-*c*,*t*-HPO is cleaved by the hydroperoxide lyase to produce *n*-hexanal.

Registry No. HPO, 71833-11-9; 13-L-*c*,*t*-HPO, 33964-75-9; 13-DL-*c*,*t*-HPO, 97672-38-3; lipoxygenase, 9029-60-1; *n*-hexanal, 66-25-1; linoleic acid, 60-33-3.

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Received for review March 15, 1985. Accepted June 5, 1985.

Formation of the Acid-Sensitive Fraction through the Interaction of Soybean Globulins and Lipids

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An acid-sensitive fraction (ASF) forming process was studied by using a model system with soybean proteins and lipids. ASF1 was formed as a result of adding glycolipids of phospholipids to β-conglycinin, precipitating the mixture at pH 4.5, and redissolving proteins in 1 M NaCl. ASF1 was gradually increased with an increase of lipids, whereas glycolipids gave more ASF1 than phospholipids. Most of the additional glycolipids migrate to the ASF1 precipitate. ASF2, which obtained as a 1 M NaCl insoluble fraction when adjusted to pH 4.5, was compared with ASF1 by using some model system consisting of soybean lecithin and soybean proteins. The yields of ASF1 were more than the yields of ASF2 in all cases and β-conglycinin gave more ASF1 and ASF2 than glycinin. ASF2 was formed from β-conglycinin but did not form from glycinin either with or without added lipids. Glycinin and mixtures of glycinin and β-conglycinin gave less ASF1 than β-conglycinin by itself; however, their yield of ASF1 increased when lipids were added. Both ASFs contain more α,α′ subunits and γ-conglycinin. The average hydrophobicity (AH) calculated from the amino acid composition of the α subunit was 940 and from γ-conglycinin was 929 cal/mol. The AH value was stronger than that of glycinin.

INTRODUCTION

Approximately 90% of the protein precipitates when an aqueous extract of defatted soybean is adjusted to pH 4.6 (Smith and Circle, 1938). Although virtually all the water-extractable soybean proteins are soluble in a phosphate buffer (Wolf and Briggs, 1956), only about 80% of this acid-precipitated protein has been found to redissolve in the buffer (Wolf and Sly, 1964). The solubility of an extracted protein appears to be modified by exposure to an acidic pH. This acid-sensitive fraction (ASF) has been obtained by Wolf and Sly (1964). The decreased solubility is attributed to the acid-sensitive proteins of the 2S and

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0021-8561/85/1433-0858$01.50/0