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Lipoxygenase-2 Isozyme Is Responsible for Generation of *n*-Hexanal in Soybean Homogenate

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The process of development of *n*-hexanal from soybean homogenate was investigated by using *Glycine max* var. Suzuyutaka (wild type) and the following lipoxygenase (L) deficient mutant seeds (L null); L-1 null, L-2 null, L-3 null, and L-1, -3 null. *n*-Hexanal was determined during the incubation of the homogenates of these seeds at 25 °C. The level of *n*-hexanal was the lowest in the L-2 null homogenate and the highest in the L-1, -3 null homogenate. After the addition of linoleic acid to the homogenates, the level of *n*-hexanal increased remarkably in the homogenates from the seeds except for L-2 null. *n*-Hexanal was scarcely generated in the L-2 null homogenate. These results suggest that L-2 isozyme is responsible for *n*-hexanal formation by using free linoleic acid as the substrate. When the soybean extract prepared from these seeds was incubated at 70 °C, *n*-hexanal formation was the lowest in the L-2 null soybean extract.

INTRODUCTION

The consumption of soy proteins as food ingredients is being encouraged, because soy protein is not only an economical resource, but also has high qualities of nutrition and functionality. However, characteristic grassy beany and green flavors build a barrier for wide utilization of soy protein. Major contributors to these flavors are the volatile carbonyl compounds which are enzymatically derived from hydroperoxides of unsaturated fatty acids (Rackis et al., 1979). Lipoxygenase (EC 1.13.11.12) catalyzes the hydroperoxidation of linoleic acid and other polyunsaturated lipids that contain a *cis,cis*-1,4-pentadiene moiety.

Soybean contains three types of lipoxygenase isozymes (lipoxygenase-1 (L-1), L-2, and L-3) which exhibit different kinetic behaviors (Galliard and Chan, 1980; Axelrod et al., 1981). Therefore, each isozyme might be expected to take part in the development of grassy beany and green flavors through a different mode of action. However, at present it is not clear how the individual isozymes participate in the generation of the flavors. Recently, soybean seeds that lack lipoxygenase isozymes were found as the result of screening a variety of seeds to get soybean with low level of the objectionable flavors (Hildebrand and Hymowitz, 1981; Hildebrand and Kito, 1984; Kitamura et al., 1983; Kitamura, 1984).

In the present study, in order to elucidate the mechanism of the development of grassy beany and green flavor from soybean, we investigated which lipoxygenase isozyme(s) was predominantly responsible for the formation of *n*-hexanal, one of major elements of soybean flavor, by using a wild type and the isozyme deficient mutants of soybeans such as L-1, L-2, L-3, and L-1, -3.

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MATERIALS AND METHODS

Soybean Cultivars. Soybeans were grown at the Iwate University Experimental Farm located in Iwate Morioka, Japan, in 1983. Normal seed (wild type) was *Glycine max* var. Suzuyutaka. L-1 deficient mutants were P.I. 408251 (L-1 null type) and a line from the cross between P.I. 408251 and Suzuyutaka. L-2 deficient mutants were P.I. 86023 (L-2 null type) and a line from the cross between P.I. 86023 and Suzuyutaka. L-3 deficient mutants were Tohoku No. 74 (L-3 null type) and a line from the cross between Tohoku No. 74 and Suzuyutaka. The first mutant of the pairs was used for the experiments at 70 °C and the latter for the experiments at 25 °C. L-1 and L-3 double deficient mutant was a line from the cross between Wasenatsu (L-3 null type) and P.I. 408251.

Reagents. (2,4-Dinitrophenyl)hydrazine, Tween 20, methyl linoleate (99%), *n*-pentanal, *n*-hexanal, *trans*-2-hexenal, *n*-heptanal, *n*-octanal, *trans*-2-octenal, *n*-nonanal, and *trans,trans*-2,4-decadienal were purchased from Nakarai Chemicals Ltd., Kyoto. (2,4-Dinitrophenyl)-hydrazones of aldehydes were synthesized by the procedure of Shriner et al. (1956). Boron trifluoride/methanol and sodium methoxide/methanol for fatty acid analysis were purchased from Applied Science Lab. Inc., PA.

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 in the experiment at 25 °C. In the experiment at 70 °C, the supernatant obtained by centrifugation (3000 rpm, 10 min, 0 °C) of the homogenate was used. The soybean extracts thus obtained were incubated at 25 °C or 70 °C for 0–60 min and were employed for the analyses.

Determination of *n*-Hexanal. *n*-Hexanal was determined as the (2,4-dinitrophenyl)hydrazone derivative with high performance liquid chromatography (HPLC) according to the methods of Selim (1977) and Reindle and Stan (1982) with some modifications. To a glass tube (3 × 9 cm) with a stopper were added 25 mL of *n*-hexane, 20 mL of 0.1% (2,4-dinitrophenyl)hydrazine in 1.1 N H_3PO_4, 0.3 N HCl, and soybean extract (0.3–1.0 mL). The mixture was shaken for 1.5 h at room temperature. After shaking, the organic phase was collected and the aqueous phase was further shaken with 25 mL of *n*-hexane for 1 h. Both the organic phases were combined, washed with water, and then evaporated to dryness under reduced pressure. The resulting residue was dissolved in 1 mL of methanol for HPLC analysis. HPLC equipment was a Hitachi 638-30 with a UV detector (Hitachi 638-0410). An integrator HP 3390 A (Hewlett-Packard) was used for the calculation of peak areas. The column used was LiChrosorb RP-18 (5μ, 4 × 250 mm) with a protective column (4 × 10 mm). The eluent consisted of acetonitrile/water/tetrahydrofuran (75:24:1, by vol). The temperature of the column was 25–30 °C and the flow rate was 1.0 mL/min. The injected volume was 20–40 μL and detection took place at 350 nm.

Measurement of Lipoxygenase Activity. L-1 and L-2, -3 activities were determined with linoleic acid as the substrate at pH 9.0 and 7.0 according to the method of Grossman and Zakut (1979), respectively. L-2, -3 activity was also measured with methyl linoleate as the substrate at pH 7.0 by the method of Hildebrand and Hymowitz (1981).

Protein Content. The protein content of soybean extracts was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard.

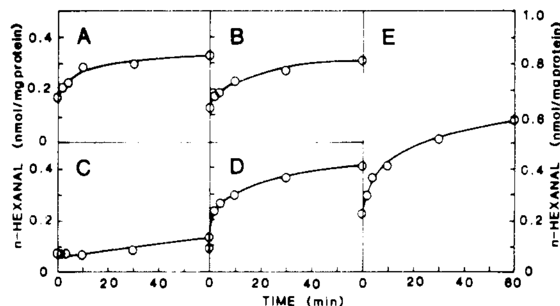

Figure 1. Changes in *n*-hexanal formation during incubation at 25 °C: (A) Suzuyutaka, (B) L-1 null, (C) L-2 null, (D) L-3 null, (E) L-1, -3 null.

Lipid Analysis. Soybeans were ground in 5% trichloroacetic acid with a mortar and pestle, and the mixture was homogenized in chloroform/methanol (2:1, v/v) with a Polytron PCU-2. The organic phase collected by centrifugation was washed with water and then evaporated by flashing N_2 gas. The residue dissolved in *n*-hexane was employed for TLC and Iatroscan analyses. Triacylglycerols, diacylglycerols, free fatty acids, and phospholipids were separated by using Merck silica gel 60 TLC plates with diethyl ether/*n*-hexane/acetic acid (50:50:1, by vol). The free fatty acids and the other lipids were methylated with boron trifluoride/methanol and with sodium methoxide/methanol, respectively. The methyl esters were determined by gas liquid chromatography (Shimadzu GC 9A) using a column packed with 10% Silar 10C, on 100–120 mesh Gaschrom Q, N_2 flow rate 45 mL/min, and with the temperature programed from 160 to 240 °C. Lipids were determined by using an Iatroscan (Iatron TH-10) analyzer with the following developing solvents: 1st developing solvent was chloroform and 2nd developing solvent was benzene/chloroform/methanol–28% NH_4OH (105:45:0.15, by vol), after methanol–NH_4OH (4:1, by vol) had been prepared. A sample was applied by using a capillary pipet on Chromarod-SII, which had been preconditioned in Iatroscan just before use. Calibration curves were different amounts of lipids above 0.5 μg. Accuracy was within 5%.

RESULTS

***n*-Hexanal Generation in Soybean Extract during Incubation at 25 °C.** The homogenates of normal type (Suzuyutaka) and L-1, L-2, L-3, and L-1, -3 null mutant seeds were incubated at 25 °C and *n*-hexanal of the homogenates was determined (Figure 1). The pH value of homogenates was 6.5–7.0 and the protein content was 10–20 mg/mL. The level of *n*-hexanal was the lowest in the homogenate of L-2 null seed (Figure 1 part C) and the highest in that of L-1, -3 null seed (Figure 1 part E). The profile of *n*-hexanal formation in the homogenates of the other seeds containing L-2 isozyme (Figure 1 parts B and D) was very close to that in Suzuyutaka (Figure 1 part A). In the case of the homogenates of seeds having L-2 isozyme (Figure 1 parts A, B, D, and E), *n*-hexanal formation increased rapidly up to 10 min and then reached a plateau. These results suggest that L-2 isozyme of three types of isozymes highly participated in *n*-hexanal formation after the soybean was ground.

Effect of Exogenous Linoleic Acid on *n*-Hexanal Formation. We attempted to examine the effect of exogenous linoleic acid on the additional formation of *n*-hexanal to *n*-hexanal from the endogenous lipids in soybean homogenate. After the homogenate of each mutant seed was incubated at 25 °C for 10 min, linoleic acid was added to the homogenate. The mixture was further in-

Table I. Effect of Exogenous Linoleic Acid on n-Hexanal Formation[a]

time after linoleic acid addition	n-hexanal, nmol/mg protein				
	Suzuyutaka	L-1 null	L-2 null	L-3 null	L-1,-3 null
5	1.3	1.4	0.12	3.0	5.5
20	1.2	1.9	0.17	3.4	4.8
	(0.30)	(0.27)	(0.10)	(0.35)	(0.52)

[a] Soybean homogenate (0.8 mL) was incubated at 25 °C for 10 min, and then linoleic acid solution (20 μL) was added. Linoleic acid (0.2%) solution containing 0.2% Tween 20 and 0.05 M phosphate buffer, pH 7.0, was used. The concentration of linoleic acid in the mixture was 128 μM. The mixture was further incubated at 25 °C for 5 and 20 min. The values in parentheses indicate those without addition of linoleic acid.

Table II. Lipid Composition of Soybean Cultivars Lacking Lipoxygenase Isozymes

	lipid class (wt %)[a]			
	TG	PL	DG	FA
Suzuyutaka	94.4	4.5	0.9	0.2
L-1 null	96.0	3.1	0.6	0.3
L-2 null	94.5	4.6	0.8	0.1
L-3 null	96.4	2.7	0.6	0.3
L-1,-3 null	93.3	6.0	0.6	0.1

[a] TG, triacylglycerol; PL, phospholipid; DG, diacylglycerol; FA, free fatty acid.

cubated at 25 °C and the n-hexanal was determined (Table I). The level of n-hexanal after the addition of linoleic acid was not increased in the homogenate of L-2 null seed, whereas it was considerably raised in the homogenates of the other four seeds (L-2 present). When the mixture was further incubated for 20 min after the addition of linoleic acid, the percentage of n-hexanal (n mol) formed to linoleic acid (n mol) added was as follows: 12% (Suzuyutaka), 23% (L-1 null seed), 39% (L-3 null seed), and 46% (L-1, -3 null seed). These results suggest that L-2 isozyme is responsible for n-hexanal formation from linoleic acid, and that a substrate of L-2 isozyme is free linoleic acid.

Lipids. Table II shows the lipid composition. In all the seeds, a large amount of triacylglycerol was contained, whereas the amount of free fatty acids was very low (Table III). Relatively little difference in lipid composition was found among the four mutant seeds. The content of linoleic acid (18:2) was the highest and that of stearic acid (18:0) was the lowest among all the seeds. The fatty acid composition of the lipid classes was similar among the mutant seeds. Distribution of linoleic acid in the free fatty acid fraction was a little lower than that of the other lipid classes.

Changes in Lipoxygenase Activity and n-Hexanal Content in Soybean Extract during Incubation at 70 °C. The soybean extract from Suzuyutaka and L-1, L-2, and L-3 null seeds was incubated at 70 °C to examine enzyme stability and to simulate change of n-hexanal formation during soy milk manufacturing. Figure 2 shows the changes in the lipoxygenase activity at 70 °C. In the case of seeds other than L-1 null mutant, L-1 activity decreased to about 50% half after a 5-min incubation and was barely detectable after a 10-min incubation. L-2 and L-3 activities completely disappeared within a 5-min incubation. These results agree with evidence that L-1 isozyme exhibits greater heat stability than L-2 and L-3 (Christopher et al., 1970). Figure 3 shows the change in n-hexanal level at 70 °C. The level of n-hexanal in the soybean extract was higher in Suzuyutaka and L-3 null seed than in L-1 and L-2 null seeds. The lowest level of n-hexanal was observed in the soybean extract of L-2 null

Table III. Fatty Acid Composition of Lipid Classes from Soybean Cultivars Lacking Lipoxygenase Isozymes

	percent				
	16:0	18:0	18:1	18:2	18:3
	Triacylglycerol Fraction				
Suzuyutaka	12.6	3.4	22.5	51.9	10.2
L-1 null	10.8	3.9	20.0	55.8	9.5
L-2 null	11.5	4.6	29.3	46.3	8.3
L-3 null	10.7	3.4	20.0	55.7	10.7
L-1,-3 null	13.4	3.2	17.5	54.0	11.8
	Phospholipid Fraction				
Suzuyutaka	17.3	2.7	5.2	65.0	9.8
L-1 null	17.1	3.2	7.5	61.4	10.8
L-2 null	18.0	3.9	6.3	61.2	10.8
L-3 null	18.4	3.3	7.2	62.6	8.5
L-1,-3 null	17.0	2.9	5.2	64.0	10.9
	Diacylglycerol Fraction				
Suzuyutaka	13.6	3.8	25.6	48.2	8.2
L-1 null	13.4	4.8	34.2	40.2	7.3
L-2 null	13.2	4.1	29.7	46.7	6.3
L-3 null	12.1	3.0	20.0	56.7	8.2
L-1,-3 null	13.9	3.0	12.4	58.6	12.1
	Free Fatty Acid Fraction				
Suzuyutaka	19.0	7.2	29.5	34.1	10.2
L-1 null	19.8	7.7	33.0	31.4	8.1
L-2 null	18.7	6.8	41.7	27.1	5.7
L-3 null	17.8	5.6	22.8	43.2	10.6
L-1,-3 null	24.7	5.2	12.3	45.8	12.0

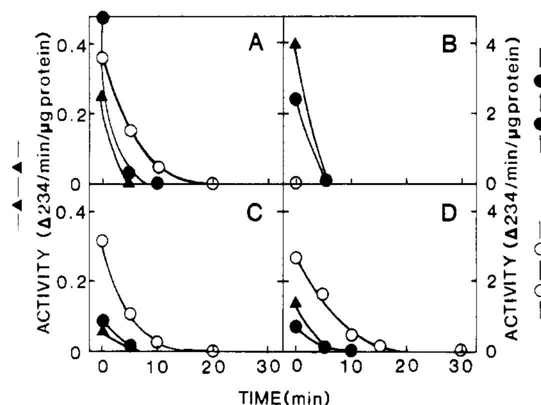

Figure 2. Changes in lipoxygenase activities during incubation at 70 °C: (A) Suzuyutaka, (B) L-1 null, (C) L-2 null, (D) L-3 null. (O-O) L-1 activity at pH 9.0, linoleic acid as the substrate; (●-●) L-2, -3 activity at pH 7.0, linoleic acid as the substrate; (Δ-Δ) L-2, -3 activity at pH 7.0, methyl linoleate as the substrate.

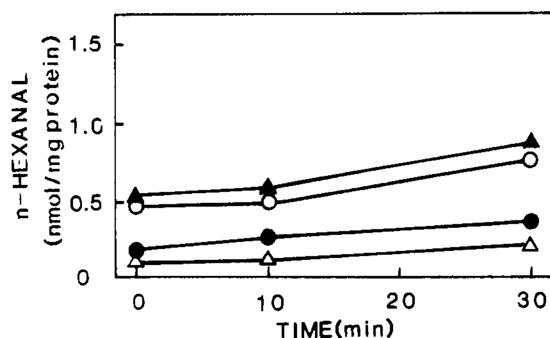

Figure 3. Changes in n-hexanal formation during incubation at 70 °C: (▲-▲) Suzuyutaka, (●-●) L-1 null, (Δ-Δ) L-2 null, (O-O) L-3 null.

seed. Hence, L-2 isozyme may be responsible for the n-hexanal formation during the heating process of soybean extract rather than L-1 isozyme. Though lipoxygenase activities in the soy milk were rarely detectable after 10 min at 70 °C in all the seeds (Figure 2), n-hexanal for-

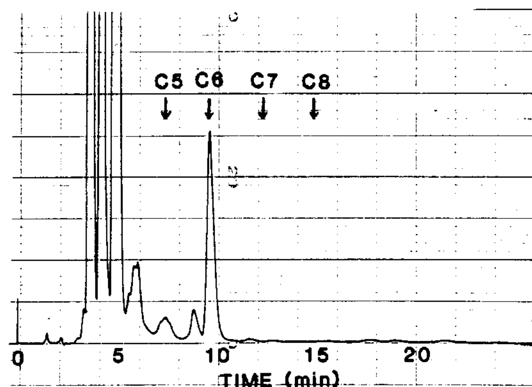

Figure 4. A profile of HPLC analysis of aldehydes produced in soybean extract of Suzuyutaka. The sample was prepared after 20 min of incubation of the extract at 25 °C. The analytical condition was described under Methods: (C_5) n-pentanal, (C_6) n-hexanal, (C_7) n-heptanal, (C_8) n-octanal.

mation increased after 10 min, implying that n-hexanal was additionally formed from unidentified lipids by enzymatic or nonenzymatic oxidation and degradation mechanism.

DISCUSSION

In the present HPLC analysis, quantitative determination of aldehydes having more than five carbons (n-pentanal) was carried out. As the results, the most abundant aldehyde which was detected was only n-hexanal in Suzuyutaka (Figure 4). The similar profiles were seen in the all other samples (data not shown). Pradel and Adda (1980) pointed out that under an acid aqueous condition hydroperoxides may be decomposed into carbonyl compounds. Since the derivatization step by (2,4-dinitrophenyl)hydrazine was carried out under an acidic condition (see Methods), there is a possibility that we determined the aldehydes, especially n-hexanal, which had been generated by acidic scission of hydroperoxides other than aldehydes originally in the samples.

Accordingly, we prepared methyl 9-hydroperoxy-10,12-octadecadienoate and methyl 13-hydroperoxy-9,11-octadecadienoate from autooxidative products of methyl linoleate by the procedure of Chan and Levett (1977). The hydroperoxides thus prepared were allowed to react with (2,4-dinitrophenyl)hydrazine under acidic condition (see Methods) in the presence or absence of soybean extract (1 mL of all the seeds). Insignificant amounts of aldehydes with more than five carbons were generated from the hydroperoxides (data not shown). Therefore, by the (2,4-dinitrophenyl)hydrazine method the successful determination of n-hexanal level in the samples was achieved. Recently, Gardner et al. (1984) reported that acid catalysis (0.1 M H_2SO_4) of 13-hydroperoxy-9,11-octadecadienoic acid in methanol–water (9:1) did not afford appreciable yields of anticipated products, such as n-hexanal and 12-oxo-9-dodecenoic acid via the known Hock rearrangement of hydroperoxides, but formed methyl 12,13-epoxy-11-methoxy-9-octadecenoates.

In soybean, linoleic acid was oxygenated by L-1 isozyme to form mainly the 13-hydroperoxide, but by L-2 and L-3 isozymes to produce a 1:1 mixture of the 9- and 13-hydroperoxides. In cucumber fruits, watermelon seedlings, tomato fruits and phaseolus leaves, the presence of hydroperoxide-cleavage enzyme (lyase) was reported (Galliard and Chan, 1980). This enzyme produces n-hexanal and *cis*-3-nonenal (or *trans*-2-nonenal) from the 13- and 9-hydroperoxides, respectively. In the present study, n-hexanal was considerably detected in all the soybean extracts with or without linoleic acid (Table I). If lyase acts on the 9-hydroperoxide of linoleic acid, the formation of *cis*-3-nonenal (or *trans*-2-nonenal) is expected to be produced. However, an insignificant amount of this aldehyde was detected even in the extracts of soybeans having L-2 and/or L-3 isozyme. We have found strong activity of hydroperoxide lyase in the normal and mutant seeds. The enzyme is specific to 13-hydroperoxide of linoleic acid (Matoba, Hidaka, Kitamura, Kaizuma, and Kito, in preparation).

From the several lines of evidence, it is likely that L-2 isozyme mainly participates in raising the n-hexanal level in the soybean homogenate at 25 °C (Figure 1). L-1 isozyme has been thought to be highly responsible for the n-hexanal formation because of its heat stability. However, the n-hexanal level in the soy milk by heat treatment at 70 °C was higher in L-1 null seed than in L-2 null seed (Figure 3), suggesting that L-2 isozyme may take part in n-hexanal formation during commercial soybean heating operations. We assume that a dominant factor to the apparent n-hexanal formation may depend on optimum pH of the isozymes (L-1, pH 9.0; L-2, L-3, pH 6.6; Axelrod et al., 1981), since the pH value of the soybean extracts was 6.5–7.0.

In soybean, the content of triacylglycerols was very high (>95%), whereas the amount of free fatty acids was extremely low (<0.3%) (Table II). The addition of free linoleic acid to the soybean homogenate caused the great increase of n-hexanal level (Table I). This suggests that a precursor of n-hexanal is predominantly free linoleic acid.

Registry No. Lipoxygenase, 9029-60-1; hexanal, 66-25-1; linoleic acid, 60-33-3.

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