Hexanal Accumulation and DETBA Value in Homogenate of Soybean Seeds Lacking Two or Three Lipoxygenase Isozymes

Yoichi Nishiba,* Shu Furuta, Makita Hajika, Kazunori Igita, and Ikuo Suda

Kyushu National Agricultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries, Nishigoshi, Kumamoto 861-11, Japan

Soybean lipoxygenases are responsible for the generation of undesirable flavors which limit wide utilization of soybeans in food products. Lipoxygenase-lacking mutants are expected to be soybeans with improved flavors. In this study, the accumulation of hexanal and the 1,3-diethyl-2-thiobarbituric acid (DETBA) value in homogenates were determined from two normal soybean cultivars ($L_{123(S)}$ and $L_{123(F)}$), three soybean cultivars with L-1 (L_1), L-2 (L_2), and L-3 (L_3), and one soybean cultivar lacking all three lipoxygenase isozymes (L_0). Hexanal accumulation in the homogenates was in the order $L_2 > L_{123(S)} > L_{123(F)} > L_1 > L_3 > L_0$. The DETBA value in the homogenates was in order $L_{123(S)}$, $L_{123(F)} > L_2$, $L_3 > L_1$, L_0. Such cultivar differences were induced after the soaked soybeans were ground or after the soybean flour was wetted. These results suggested that L_0 could become a superior soybean for food ingredients, concerning flavors originating from lipoxygenases.

Keywords: *Soybean; lipoxygenase isozymes; hexanal; DETBA value; lipid peroxidation; food ingredient; Glycine max*

INTRODUCTION

Soybean protein is an important food ingredient, because it is an economical food with a high quality of nutrition and functionality. However, characteristic undesirable grassy-beany flavors hinder its wide utilization. Major contributors to these flavors are the volatile carbonyl compounds such as *n*-hexanal, which are formed by hydroperoxidation of unsaturated fatty acids through the action of lipoxygenase and subsequent cleavage of the product by hydroperoxide lyase (Rackis et al., 1979; Matoba et al., 1985a,b; Hildebrand, 1989). Treatments such as heat or extraction with an organic solvent have been used to improve the flavors of soybean products (Wolf, 1975; Rackis et al., 1979; Sessa, 1979) but are expensive and not entirely adequate.

Another approach for improving the flavors is to develop soybeans in which there is little or no lipoxygenase activity. Normal soybean seed contains three lipoxygenase isozymes called L-1, L-2, and L-3, respectively (Axelrod et al., 1981). In the early 1980s, L-1 null (Hildebrand and Hymowitz, 1981), L-2 null (Kitamura et al., 1985; Davies and Nielsen, 1986), and L-3 null (Kitamura et al., 1983) soybeans were identified, and the finding of double L-1 and L-3 null and double L-2 and L-3 null soybeans followed (Kitamura et al., 1985). There are many studies dealing with hexanal production using these lipoxygenase-lacking cultivars. Matoba et al. (1985a) reported that L-2 was largely responsible for the generation of hexanal. Davies et al. (1987) also showed that genetic elimination of L-2 was effective in improving the flavor of soybean products. Hildebrand et al. (1990) and Moreira et al. (1993) indicated that L-3 caused a decrease in hexanal production. However, these cultivars still appear to show an appreciable amount of hexanal in the homogenates. Such information led us to postulate that the use of soybeans lacking all three lipoxygenase isozymes in the seed (triple-null soybean) might be the most effective solution to reduce undesirable flavors associated with soybean products and thereby to increase their consumer acceptability.

Recently, Hajika et al. (1991) succeeded in inducing the triple-null soybean by γ-ray irradiation, and they further obtained the double L-1 and L-2 null soybean by segregation of F_2 seeds from the cross between a normal soybean and a triple-null soybean (Hajika et al., 1992). To elucidate whether the triple-null soybean has the ability to improve the flavor, we here examined the 1,3-diethyl-2-thiobarbituric acid (DETBA) value and hexanal accumulation in aqueous homogenates obtained from the triple-null soybean and compared the results with those of other soybeans. The DETBA values were determined to estimate lipid peroxidation products (Suda et al., 1994), which are precursors of *n*-hexanal and other volatile carbonyl compounds (Hildebrand, 1989) and are also speculated to be related to some diseases (Ames, 1983; Haumann, 1993). We used homogenates prepared both by wetting soybean flour or by disrupting soaked soybeans as samples to obtain information applicable to soybean processing.

MATERIALS AND METHODS

Soybean Cultivars. Soybean [*Glycine max* (L.) Merr.] cultivars used in this study were as follows. Suzuyutaka and Fukuyutaka were normal soybean cultivars containing all three types of lipoxygenase isozymes in the seeds (abbreviated $L_{123(S)}$ and $L_{123(F)}$ from the remaining-isozyme phenotype). Yumeyutaka (L_1), Kanto 102 (L_2), and Kyushu 119 (L_3) were cultivars containing only one isozyme, L-1, L-2, or L-3, respectively. Kyushu 111 (L_0) was a cultivar lacking all three types of lipoxygenase isozymes. The lipoxygenase-lacking cultivars were derivatives of Suzuyutaka. All seeds were harvested at Kyushu National Agricultural Experiment Station in 1992–1993.

Electrophoresis. The presence or absence of the lipoxygenase isozymes in soybean seeds was analyzed using distal portions of the seeds by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Kitamura (1984).

Preparation of Homogenate from Soybean Flour or Soaked Soybean Seeds. Soybean flour was prepared by grinding four or five seeds in a coffee mill. A suspension of

* Author to whom correspondence should be addressed (fax 81-96-249-1002).

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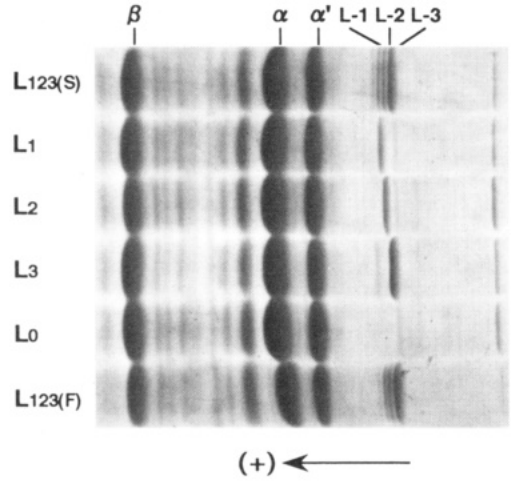

Figure 1. Analysis of lipoxygenase isozymes in soybean seeds by SDS–polyacrylamide gel electrophoresis: L-1, lipoxygenase 1; L-2, lipoxygenase-2; L-3, lipoxygenase-3; α, α', and β subunits of 7S globulin (β-conglycinin).

0.5 g of flour in 10 mL of ice-cooled distilled water was homogenized with a Polytron homogenizer (Kinematica, Switzerland) under cooling in an ice bath. Soybean seeds were soaked in distilled water (0.5 g/10 mL) at 4 °C overnight and homogenized in the same manner as the soybean flour.

Measurement of Lipoxygenase Activity. The homogenate prepared from soaked soybean seeds was allowed to stand for 30 min at 4 °C and was then centrifuged (10000*g*, 10 min, 4 °C). The supernatant was used for measuring lipoxygenase activity within 1 h. The assay was performed by determining conjugated diene formation at 234 nm, pH 9.0 for L-1, pH 6.6 for L-2 plus L-3, and determining keto–diene formation at 280 nm, pH 6.6 for L-3, using linoleate as substrate (Wang et al., 1990). Units of L-1, L-2, and L-3 activity are indicated as micromoles of products per minute.

Measurement of DETBA Value. The homogenate prepared from soybean flour or soaked soybeans was kept at 4 °C in a screw-capped tube. After an appropriate time, an aliquot of each homogenate was added to 1.5 volumes of 20 mM butylated hydroxytoluene in ethanol to stop the enzyme reaction and subjected to DETBA assay as described previously (Suda et al., 1994). The DETBA values were expressed as nanomoles of malondialdehyde per gram of soybean seeds. In the case of measurement of the initial DETBA value, soybean flour or soaked soybeans were added directly to 20 mM butylated hydroxytoluene in ethanol and homogenized.

Determination of *n*-Hexanal. The homogenate prepared from soybean flour was kept at 4 °C. After an appropriate time, an aliquot of the homogenate was employed for determination of *n*-hexanal. *n*-Hexanal was determined as the 2,4-dinitrophenylhydrazone derivative with high-performance liquid chromatography (HPLC) according to the method of Matoba et al. (1985a) with *n*-hexanal 2,4-dinitrophenylhydrazone (Tokyo Kasei Kogyo Co., Tokyo, Japan) as the standard. The HPLC equipment was a Jasco PRETOP 2 system with a mulch channel detector (Jasco MD910). HPLC analysis was performed under the following conditions: column, LiChrosorb RP-18 (5 μm particle size, 250 × 4 mm i.d.; GL Sciences Inc., Tokyo, Japan); mobile phase, acetonitrile/water/tetrahydrofuran (75:24:1 by vol); flow rate, 1.0 mL/min; temperature, 30 °C; detection, 363 nm. In the case of measurement of the initial hexanal level, soybean flour was added directly to phosphoric acid/HCl solution and homogenized.

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

RESULTS

Cultivar Differences in Lipoxygenase Isozymes in Soybean Seeds. Figure 1 shows a typical SDS–PAGE pattern of the lipoxygenase isozymes in two normal soybean seeds ($L_{123(S)}$ and $L_{123(F)}$), three double-null soybean seeds (L_1, L_2, and L_3), and one triple-null soybean seed (L_0). We used these soybean seeds for all subsequent experiments. The presence or absence of individual lipoxygenase isozymes contained in each soybean seed was also confirmed by spectrophotometric assay (Table 1). The L_1 and L_0 were characterized as soybeans with weak lipoxygenase activities at neutral pH, compared with the others.

Table 1. Cultivar Differences in Lipoxygenase Isozyme Activities in Soybean Seeds

cultivar	activity (units/mg of protein) L-1[a]	L-2 + L-3[b]	L-3[c]
Suzuyutaka ($L_{123(S)}$)	0.92[d]	0.90	0.28
Yumeyutaka (L_1)	1.57	0.09	0.07
Kanto 102 (L_2)	0.01	0.54	0.03
Kyushu 119 (L_3)	0.01	0.35	0.32
Kyushu 111 (L_0)	0.00	0.05	0.05
Fukuyutaka ($L_{123(F)}$)	1.42	1.40	0.33

[a] Measured at 234 nm, pH 9.0. [b] Measured at 234 nm, pH 6.6. [c] Measured at 280 nm, pH 6.6. [d] Results expressed as means of four experiments.

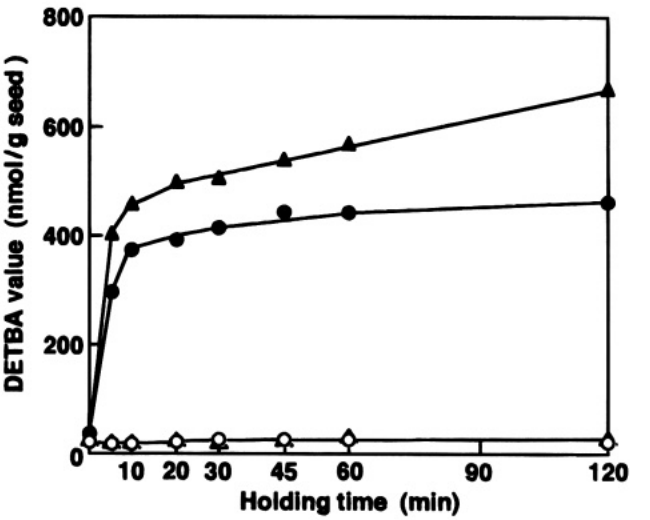

Figure 2. DETBA value in homogenates as a function of holding time at 4 °C after homogenization. Soaked soybeans or soybean flour was homogenized with distilled water and kept at 4 °C. After an appropriate time, the DETBA value was determined. In the case of samples at 0 min, soaked soybeans or soybean flour was homogenized with butylated hydroxytoluene in ethanol and subjected to DETBA assay. Results are expressed as means of two experiments: (●) $L_{123(S)}$, soaked soybeans; (○) L_0, soaked soybeans; (▲) $L_{123(S)}$, soybean flour; (△) L_0, soybean flour.

Cultivar Differences in DETBA Value. The homogenates of $L_{123(S)}$ and L_0 soybean seeds were prepared from soaked soybeans and soybean flour, and the DETBA values of the homogenates were determined as a function of holding time at 4 °C after homogenization (Figure 2). In $L_{123(S)}$ soybean homogenates, the DETBA value increased rapidly up to 10 min and then slowed. On the contrary, its increase was not observed in the L_0 soybean homogenate. Figures 3 and 4 show the cultivar differences in DETBA values in two types of homogenates. Although the DETBA values in soaked soybeans without grinding or in soybean flour without wetting were almost equivalent among six cultivars (Figures 3A and 4A), the cultivar difference appeared when the homogenate samples were kept for 60 min at 4 °C (Figures 3B and 4B). In homogenates prepared by grinding soaked soybeans, two normal soybeans ($L_{123(S)}$ and $L_{123(F)}$) had the highest DETBA value, followed by L_2, L_3, L_1, and L_0 (Figure 3). This order nearly correlated with the lipoxygenase activities at

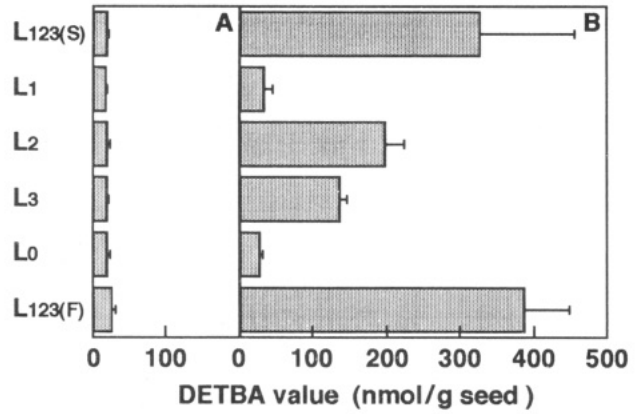

Figure 3. Cultivar differences in DETBA value in homogenates prepared from soaked soybeans. Experimental conditions were as described in Figure 2 except that the homogenates prepared from soaked soybeans were kept for 0 (A) or 60 min (B) at 4 °C. Results are expressed as means ± standard errors of four experiments.

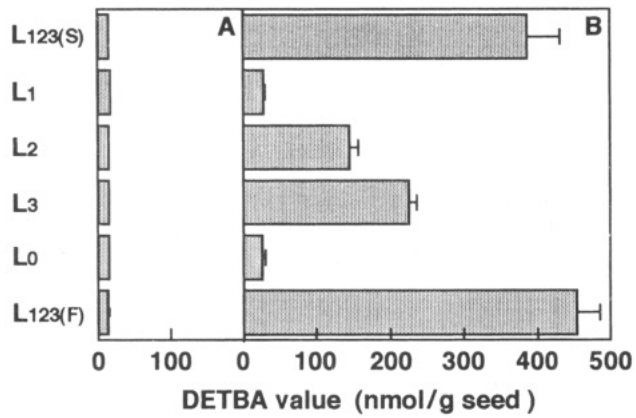

Figure 4. Cultivar differences in DETBA value in homogenates prepared from soybean flour. Experimental conditions were as described in Figure 2 except that the homogenates prepared from soybean flour were kept for 0 (A) or 60 min (B) at 4 °C. Results are expressed as means ± standard errors of four experiments.

neutral pH in extracts obtained from soaked soybean homogenates (Table 1). The cultivar differences in DETBA values in homogenates prepared by wetting soybean flour were similar to those of homogenates from soaked soybeans, except for the order of L_2 and L_3 (Figure 4).

Cultivar Differences in Hexanal Accumulation. The time course study showed rapid initial accumulation and subsequent slowing in accumulation of hexanal in $L_{123(S)}$ soybean flour homogenates and slow accumulation throughout the holding time in L_0 soybean flour homogenates (Figure 5). Figure 6 shows the cultivar differences in hexanal accumulation in homogenates prepared from soybean flour. Like the DETBA value, the cultivar differences appeared only when the homogenates were kept for 60 min at 4 °C. However, hexanal accumulation was in order $L_2 > L_{123(S)} > L_{123(F)} > L_1 > L_3 > L_0$.

DISCUSSION

The present study showed that cultivar differences in DETBA value and hexanal accumulation did not appear in the soaked soybeans without grinding or the soybean flour without wetting, but cultivar differences did appear with the action of lipoxygenase when they were subjected to such treatments (Figures 3, 4, and 6). This means that if soaked soybeans or soybean flour

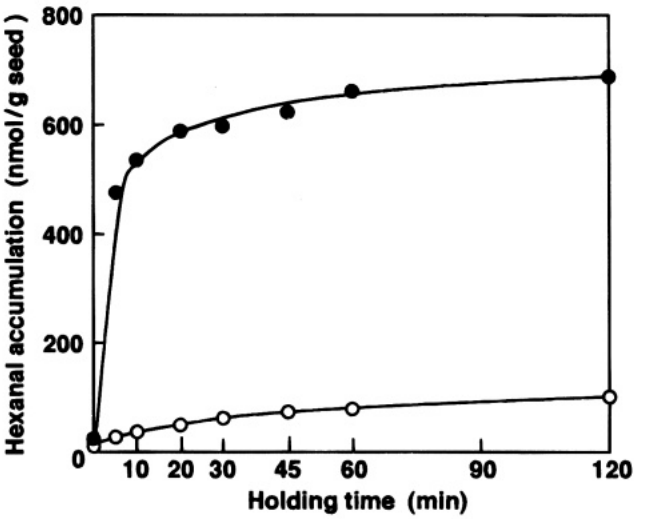

Figure 5. Hexanal accumulation in homogenates as a function of holding time at 4 °C after homogenization. Soybean flour was homogenized with distilled water and kept at 4 °C. After an appropriate time, the amount of hexanal accumulated in the homogenates was determined. In the case of samples at 0 min, soybean flour was directly homogenized in phosphoric acid/HCl solution. Results are expressed as means of two experiments: (●) $L_{123(S)}$; (○) L_0.

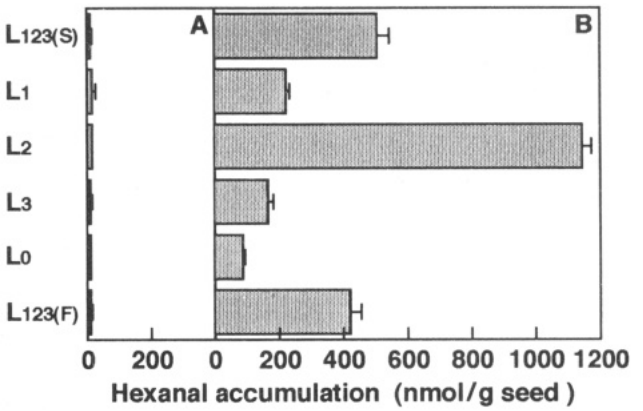

Figure 6. Cultivar differences in hexanal accumulation in homogenates prepared from soybean flour. Experimental conditions were as described in Figure 5 except that the homogenates were kept for 0 (A) or 60 min (B) at 4 °C. Results are expressed as means ± standard errors of four experiments.

is processed by heating or boiling before the attack of lipoxygenase on unsaturated fatty acids contained in soybean seeds, the superior character of lipoxygenase-lacking soybeans cannot be observed. Actually, we could not find differences in DETBA values between $L_{123(S)}$ and L_0 soybeans when the soaked soybeans were boiled or when the soybean flour was heated in a dry oven (data not shown). However, the effect of eliminating lipoxygenases on DETBA value was observed in soy milk which was made by grinding soaked soybean seeds with hot (or cold) water and then filtering. The L_0 soy milk prepared with cold water was superior to that of $L_{123(S)}$ in DETBA value by a factor of about 10 (e.g., L_0 = 3.5 nmol/mL, $L_{123(S)}$ = 40.9 nmol/mL). On the other hand, when hot-water processing was used, the $L_{123(S)}$ bean product was improved but the L_0-derived product was still 2–3 times better (e.g., L_0 = 3.9 nmol/mL, $L_{123(S)}$ = 12.0 nmol/mL). Perhaps this treatment with hot water is ineffective in completely inactivating lipoxygenases. As indicated, lipoxygenase-lacking soybeans should be used in manufacturing processes in which lipoxygenase action is unavoidable. If food producers pay attention to this point, lipoxygenase-lacking soy-

beans will become good ingredients for making soybean products with high quality.

In this study, we used two parameters, DETBA value and hexanal accumulation, for selection of the most suitable soybean seeds as a food ingredient. Determination of the amount of hexanal accumulated in the homogenates is important, because hexanal has a low olfactory threshold and creates great difficulties in the production of acceptable food products. Measurement of the DETBA value used as an index of lipid peroxidation products is also important, because lipid peroxidation products are precursors of *n*-hexanal and other carbonyl compounds (Hildebrand, 1989). Furthermore, consumers have recently requested healthful foods; thus, lipid peroxidation in food, which also leads to toxic substances and nutritional damage, should be avoided. Some studies suggest that the lipid peroxidation products may be implicated in several diseases (Ames, 1983; Haumann, 1993).

Among various lipoxygenase-lacking soybeans, L_0 soybeans have the lowest levels of both DETBA values and hexanal accumulation. Therefore, this soybean can be expected to become an excellent food ingredient.

Compared to normal soybeans, the L_1 soybeans had low levels of both DETBA value and hexanal accumulation. The low levels of both parameters were probably due to the weak lipoxygenase activities at neutral pH (Table 1). We confirmed that the pH of the L_1 homogenates used was 6.3–6.5, while the optimum pH for the L-1 isozyme is around pH 9.0. Thus, the L_1 soybeans may be suitable for a food ingredient judging from the low level of the DETBA value, if alkaline pH conditions are avoided in food product preparation.

It is well-known that L-2 is largely responsible for hexanal production (Matoba et al., 1985a). We also confirmed in this study that the L_2 soybeans have a high hexanal accumulation and an intermediate DETBA value, indicating that L_2 soybeans may be unsuitable for foodstuffs unless processing modifications (e.g., heat inactivation of lipoxygenase) are made.

The level of hexanal accumulated in L_{123} soybean homogenate was lower than in L_2 soybean homogenate. Also, the level in L_3 soybean homogenate was lower than in L_1 soybean homogenate, though the DETBA value was higher than that in the L_1 soybean homogenate. This is probably due to the effect of L-3 in reducing hexanal production as described by Hildebrand et al. (1990) and Moreira et al. (1993). Therefore, judging only from the low accumulation of hexanal, L_3 soybeans may be suitable for foodstuffs.

In the present study, we clearly demonstrated that L_0 had the lowest levels of both parameters of DETBA value and hexanal accumulation among two or all three lipoxygenase-lacking soybeans. Therefore, L_0 soybeans lacking all three lipoxygenase isozymes in seeds appear to be superior as a food ingredient from the standpoints of improvement in both flavor and human health safety.

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