Simple and Rapid Method for the Selective Detection of Individual Lipoxygenase Isozymes in Soybean Seeds

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

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

A simple and rapid spectrophotometric and visual judging method was developed for the selective detection of individual lipoxygenase isozymes (L-1, L-2, and L-3) in soybean [*Glycine max* (L.) Merrill] seeds, based on the bleaching activities of these isozymes in contact with methylene blue and β-carotene. The method consists of three procedures designated tests I–III. The tests are performed by mixing soybean extract samples or wetted soybean flour with each dye–substrate of test I for the L-1 isozyme, test II for the L-2 isozyme, and test III for the L-3 isozyme, whereupon the presence of individual isozymes contained in the soybean is determined spectrophotometically or visually within 10 or 5 min, respectively. In the visual judging method available as a routine screening test, the total amount of soybean flour required for the three tests is only about 10 mg.

Keywords: *Soybean; lipoxygenase; isozyme; mutant; bleaching; methylene blue; β-carotene; screening; Glycine max*

INTRODUCTION

Normal soybean seeds contain three lipoxygenase isozymes, called lipoxygenase 1 (L-1), lipoxygenase 2 (L-2), and lipoxygenase 3 (L-3) (Axelrod et al., 1981). The isozymes are responsible for the generation of grassy-beany and bitter flavors which hinder wider utilization of soybeans as food ingredients (Wolf, 1975; Rackis et al., 1979).

Genetic elimination of lipoxygenase from soybean seeds has attracted attention as a useful approach to reduce such undesirable flavors. So far, five types of lipoxygenase-lacking cultivars, L-1 null (Hildebrand and Hymowitz, 1981, 1982), L-2 null (Kitamura et al., 1985; Davies and Nielsen, 1986), L-3 null (Kitamura et al., 1983), double L-1 and L-3 null (Kitamura et al., 1985; Kitamura, 1991), and double L-2 and L-3 null (Kitamura et al., 1985, 1992; Kitamura, 1991) type soybeans, have been reported. More recently, the remaining types, triple L-1, L-2, and L-3 null (Hajika et al., 1991) and double L-1 and L-2 null type soybeans (Hajika et al., 1992), have been produced. To stimulate development of all types of lipoxygenase-lacking cultivars, various trials are in progress to obtain lipoxygenase-lacking cultivars with superior agricultural traits. However, the lack of a lipoxygenase isozyme detection method available for routine screening limits the usefulness of these approaches.

The presence or absence of three lipoxygenase isozymes can be readily and accurately determined by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) method (Kitamura, 1984). Lipoxygenase isozyme activities are also assayed by absorbance changes at 234 and 280 nm (Axelrod et al., 1981; Wang et al., 1990) or by polarographic (Axelrod et al., 1981; Omura et al., 1986) and immunological techniques (Yabuuchi et al., 1982; Mack et al., 1987). However, these four methods are time-consuming and require a number of manipulations for determination.

On the other hand, a dye solution bleaching method is the most powerful detection technique applicable to a visual judging method. There are some studies dealing with dye solution bleaching by soybean lipoxygenases. Toyosaki (1992) showed that commercial L-1 bleached methylene blue, although it was uncertain whether other isozymes did so. Kikuchi and Kitamura (1987) reported three tests and two versions for the detection of lipoxygenase isozymes by skillful use of the different action among the carotene bleaching activities of three lipoxygenase isozymes; however, the tests involved complex procedures and careful handling for the detection of the L-1 or L-2 isozyme in soybean seeds lacking the L-3 isozyme. Also, Hammond et al. (1992) reported color tests based on the oxidation of iodine–starch for the L-1 isozyme or of ferrous thiocyanate for the L-2 and L-3 isozymes. However, the tests had a weakness in that the test for the L-3 isozyme was valid only in the absence of the L-2 isozyme and also required time-consuming manipulations.

Here, we report a simple and rapid spectrophotometric method for the selective detection of individual lipoxygenase isozymes in soybean seeds. The method is based on the differential bleaching ability of L-1, L-2, and L-3 isozyme in contact with methylene blue and β-carotene. Tests I, II, and III can detect the presence of the L-1, L-2, and L-3 isozymes contained in soybean seeds, respectively. We also report a visual judging method with a slight modification of the spectrophotometric method, which is available as a routine screening test.

MATERIALS AND METHODS

Soybean Cultivars. Two normal soybean and seven lipoxygenase-lacking cultivars were used in this study (Table 1). Suzuyutaka and Fukuyutaka were normal soybean cultivars containing L-1, L-2, and L-3 isozymes (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. L_{12}, L_{13}, and L_{23} were cultivars containing two isozymes, L-1 and L-2, L-1 and L-3, and L-2 and L-3, respectively. Kyushu 111 (L_0) was a cultivar lacking all three types of lipoxygenase isozymes. The presence or absence of lipoxygenase isozymes in their soybean seeds was confirmed by SDS–PAGE and

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

0021-8561/95/1443-0742$09.00/0

Table 1. Soybean Cultivars Used in This Study and Their Abbreviations

cultivar	abbrev[a]	phenotype[b] L-1	L-2	L-3
Suzuyutaka	$L_{123(S)}$	+	+	+
Yumeyutaka	L_1	+	−	−
Kanto 102	L_2	−	+	−
Kyushu 119	L_3	−	−	+
Kyushu 111	L_0	−	−	−
Fukuyutaka	$L_{123(F)}$	+	+	+
L-3 null	L_{12}	+	+	−
L-2 null	L_{13}	+	−	+
L-1 null	L_{23}	−	+	+

[a] Abbreviated on the basis of the phenotype of lipoxygenase isozymes remaining in the soybean seeds. (S), Suzuyutaka; (F), Fukuyutaka. [b] (+), presence; (−), absence.

spectrophotometric methods (Nishiba et al., 1995). The lipoxygenase-lacking cultivars were derivatives of Suzuyutaka. These soybeans were harvested at Kyushu National Experiment Station 1992−1993 and powdered by grinding with a coffee mill or by drilling into the cotyledons without injury to the hypocotyl.

Reagents. Linoleic acid (99%) and β-carotene (80−90% β-isomer, from carrot) were obtained from Sigma Chemical Co., St. Louis, MO. Methylene blue and dithiothreitol were purchased from Wako Pure Chemical Industries Co., Osaka, Japan. The other chemicals were of analytical grade.

Preparation of Soybean Extract. Soybean flour of each cultivar was homogenized with 98 volumes of ice-cooled distilled water in a Polytron homogenizer (Kinematica, Switzerland) at a level 4 speed and allowed to stand for 1 h at 4 °C. The supernatant obtained by centrifugation (10000*g*, 10 min, 4 °C) was used as the soybean extract sample for the test within a few hours.

Preparation of Substrate. Sodium linoleate substrate was prepared from linoleic acid as described by Axelrod et al. (1981). Briefly, 70 mg of linoleic acid and 70 mg of Tween 20 were homogenized in 4 mL of autoclaved distilled water by drawing the mixture back and forth in a Pasteur pipet, avoiding air bubbles. To obtain a clear solution, about 0.55 mL of 0.5 N NaOH was added, and the solution was made up to 25 mL total volume with autoclaved distilled water. The solution (2 mL aliquots) was placed into vials, covered with N_2 gas, and stored at −20 °C until use.

Preparation of β-Carotene at 50% Saturation in Acetone. About 10 mg of β-carotene was dissolved in 10 mL of acetone. After being vigorously stirred several times and centrifuged, the orange-colored supernatant was diluted with the same volume of acetone and stored in a brown vial at 4 °C. This solution was prepared daily.

Spectrophotometric Method for Detecting Lipoxygenase Isozymes. *Standard Procedure of Test I.* The reaction mixture contained 0.5 mL of 200 mM sodium borate buffer (pH 9.0), 0.1 mL of 100 μM methylene blue, 0.1 mL of 10 mM sodium linoleate substrate, 0−0.3 mL of soybean extract sample, and distilled water to a final volume of 1.0 mL. The reaction was initiated by the addition of the soybean extract sample, and the absorbance at 660 nm was recorded with a spectrophotometer (Beckman DU70) for 5 min at 25 °C.

Standard Procedure of Test II. The reaction mixture contained 0.4 mL of 200 mM sodium phosphate buffer (pH 6.0), 0.1 mL of 100 μM methylene blue, 0.1 mL of 200 mM dithiothreitol in 200 mM sodium phosphate buffer (pH 6.0) (prepared every few hours), 0.1 mL of acetone, 0.1 mL of 10 mM sodium linoleate substrate, 0−0.2 mL of soybean extract sample, and distilled water to a final volume of 1.0 mL. The reaction was initiated by the addition of the soybean extract sample, and the absorbance at 660 nm was measured for 10 min at 25 °C.

Standard Procedure of Test III. The reaction mixture contained 0.5 mL of 200 mM sodium phosphate buffer (pH 6.6), 0.1 mL of β-carotene at 50% saturation in acetone, 0.1 mL of 10 mM sodium linoleate substrate, 10 μL of soybean extract obtained from Kanto 102 containing only the L-2 isozyme

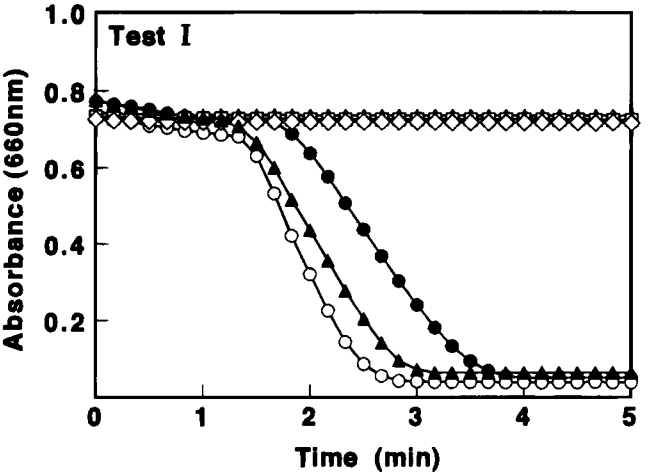

Figure 1. Response of extracts obtained from two normal soybean and four lipoxygenase-lacking cultivars to test I. Soybean extract (20 μL) obtained from $L_{123(S)}$ (●), $L_{123(F)}$ (▲), L_1 (○), L_2 (△), L_3 (□), and L_0 (◇) was added to the test I reaction system.

alone, 0−0.29 mL of soybean extract sample, and distilled water to a final volume of 1.0 mL (test III). The β-carotene solution in the cuvette should be mixed quickly with the other reaction mixture components excluding the soybean extracts; otherwise, one may not obtain a clear solution. The reaction was initiated by the addition of the soybean extract sample, and the absorbance at 452 nm was measured for 5 min at 25 °C. In another experiment, soybean extract obtained from Kanto 102 was omitted from the test III reaction mixture (incomplete test III).

Visual Judging Method for Detecting Lipoxygenase Isozymes. Test I was conducted as follows: (i) 2.5 mg of soybean flour was weighed into a test tube; (ii) 0.5 mL of distilled water was added, and the mixture was stirred lightly and allowed to stand for 3−10 min; (iii) dye−substrate was prepared by mixing 25 mL of 200 mM sodium borate buffer (pH 9.0), 5 mL of 100 μM methylene blue, 5 mL of 10 mM sodium linoleate substrate, and 5 mL of distilled water in a 100 mL glass-stoppered bottle (for 20 samples); (iv) 2 mL of the dye−substrate of (iii) was added to the test tube with a Eppendorf Repeater pipettor equipped with Combitips; (v) after 3 min, the color of the solution was checked visually.

Test II involved the same procedures as test I, except for the following modifications: (i) 5.0 mg of flour was used; (iii) 154.25 mg of dithiothreitol was weighed into a 100 mL glass-stoppered bottle, and then 25 mL of 200 mM sodium phosphate buffer (pH 6.0), 5 mL of 100 μM methylene blue, 5 mL of acetone, and 5 mL of 10 mM sodium linoleate substrate were added, and the mixture was then swirled in the bottle (dye−substrate for 20 samples); (v) after 5 min, the color was checked.

Test III involved the same procedures as test I, except for the following modifications: (ii) soybean extract containing L-2 isozyme alone was prepared by the centrifugation of 1 mg/mL Kanto 102 homogenate in distilled water, and 0.5 mL of it was added instead of distilled water; (iii) 25 mL of 200 mM sodium phosphate buffer (pH 6.6), 5 mL of 10 mM sodium linoleate substrate, and 5 mL of distilled water were mixed and added to a 100 mL glass-stoppered bottle containing 5 mL of β-carotene at 50% saturation in acetone; the mixture was immediately shaken vigorously (dye−substrate for 20 samples).

Measurement of Oxidation−Reduction Potential. Change in the oxidation−reduction potential (ORP) in solution was monitored with a pH/ORP meter (Horiba F-14, Kyoto, Japan) equipped with a platinum combination electrode.

RESULTS AND DISCUSSION

Test I for the Detection of the L-1 Isozyme. Figure 1 shows a typical response of various soybean

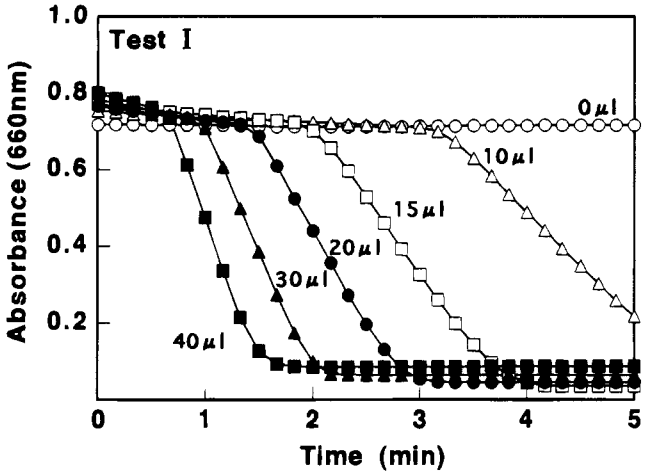

Figure 2. Methylene blue bleaching by L_1 soybean extracts. Indicated amounts of L_1 soybean extracts were added to the test I reaction system.

extracts to test I. Three soybean extracts containing the L-1 isozyme ($L_{123(S)}$, $L_{123(F)}$, and L_1 soybean extracts) bleached methylene blue within a few minutes, when 20 μL of soybean extract was added to the reaction mixture. The other soybean extracts lacking the L-1 isozyme showed no bleaching activity toward methylene blue even if 200 μL of each soybean extract was added to the reaction mixture. These results indicated that test I could selectively detect the L-1 isozyme contained in soybean extracts.

Toyosaki (1992) proposed that methylene blue bleaching occurred as a result of the sequence of production of the lipid radical R$^\bullet$ and the peroxy radical ROO$^\bullet$. Another simple explanation is that a redox reaction is likely involved in this bleaching reaction, because methylene blue is known to be a redox indicator. Therefore, we examined the relationship between methylene blue bleaching activity and the change in ORP in solution. Methylene blue bleaching occurred rapidly as the added amount of L_1 soybean extract increased (Figure 2). Similar results were also obtained in two L_{123} soybean extracts. On the other hand, the ORP (mV) fell linearly after a short lag time depending on the L-1 isozyme activity (data not shown). As shown in Figure 3, a linear relationship existed between the time required for the bleaching of methylene blue to begin and the time required for the dropping of ORP to begin. This good correlation suggested the involvement of a redox reaction in test I.

Test II for the Detection of the L-2 Isozyme. We next tried to find the experimental conditions under which either the L-2 or the L-3 isozyme, but not the L-1 isozyme, could bleach methylene blue. It is well-known that the L-2 and L-3 isozymes have strong activity toward a sodium linoleate substrate at neutral pH, unlike the L-1 isozyme (Axelrod et al., 1981). However, methylene blue bleaching in response to either the L-2 or L-3 isozyme was not observed with the change in pH alone in the test I reaction mixture (from pH 9.0 to pH 6.0–7.0), indicating that the addition of other factors was necessary for its detection. As a result of many efforts, we finally developed a test detecting the L-2 isozyme only, called test II. The reaction system consisted of phosphate buffer (pH 6.0), methylene blue, acetone, dithiothreitol, sodium linoleate, and the soybean extract sample.

Each of the components of the system was required for appreciable observation of methylene blue bleaching

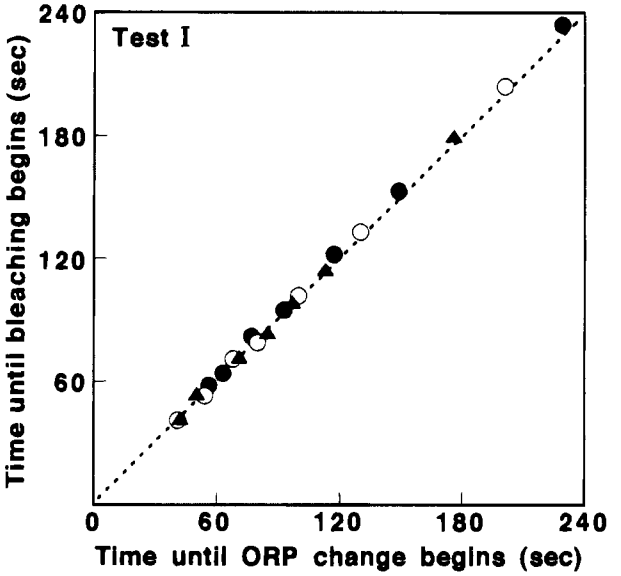

Figure 3. Correlation between the time required for the bleaching of methylene blue to begin and the time required for the dropping of the oxidation–reduction potential to begin. Ten to fifty microliters of soybean extracts obtained from $L_{123(S)}$ (●), $L_{123(F)}$ (▲), and L_1 (○) were used.

Table 2. Effect of Deletion of Components from the Test II System on the Methylene Blue Bleaching

system	methylene blue bleaching[a]
complete system[b]	−0.232
dithiothreitol omitted	+0.130
acetone omitted	−0.090
linoleate omitted	+0.037

[a] Each value was calculated by subtracting A_{660} at 10 min from A_{660} at 0 min. [b] Complete reaction mixture contained 100 mM phosphate buffer, pH 6.0, 10 μM methylene blue, 20 mM dithiothreitol, 10% acetone, 1 mM sodium linoleate substrate, and 50 μL/mL L_2 soybean extract.

with the L_2 soybean extract (Table 2). Dithiothreitol was the most essential factor among them. Prior to the development of test II, we found that when the pH in the reaction mixture was neutral, the redox balance leaned toward the oxidized state compared to the condition at pH 9.0. Thus, we used dithiothreitol to lower the redox potential inducing methylene blue bleaching. Although methylene blue bleaching occurred with the addition of dithiothreitol at a concentration beyond 2 mM, 20 mM dithiothreitol was preferably used for detection both visually and by spectrophotometer. This stimulating effect was also observed with the addition of fresh cysteine or glutathione solution; however, these agents were not suitable for obtaining reproducible results, because of their gradual inactivation by air oxidation. A study of pH dependence showed that pH 6.0 was the most favorable. At pH 6.8, the soybean extracts containing the L-1 isozyme also slightly bleached slightly methylene blue, and at pH below pH 6.0, the bleaching activity of the soybean extracts containing the L-2 isozyme was diminished probably because of insolubility of the substrate. Acetone was added to dissolve the substrate in the reaction mixture at pH 6.0.

Under such complete experimental conditions, the response of various soybean extracts to test II was examined. As shown in Figure 4, three soybean extracts containing the L-2 isozyme ($L_{123(S)}$, $L_{123(F)}$, and L_2 soybean extracts) bleached methylene blue, while the

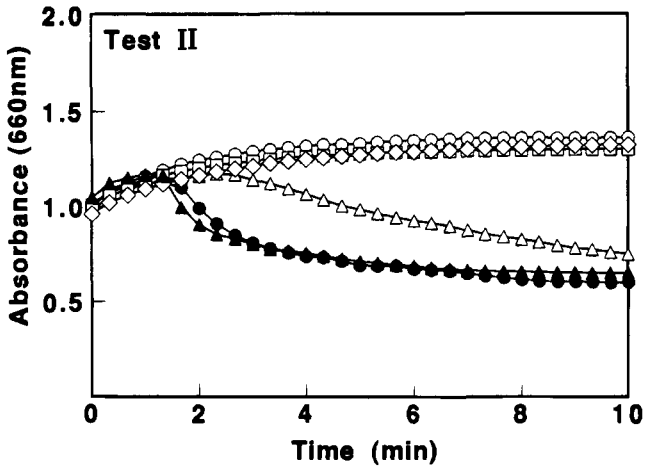

Figure 4. Response of extracts obtained from two normal soybean and four lipoxygenase-lacking cultivars to test II. Soybean extract (50 μL) obtained from $L_{123(S)}$ (●), $L_{123(F)}$ (▲), L_1 (○), L_2 (△), L_3 (□), and L_0 (◇) was added to the test II reaction system.

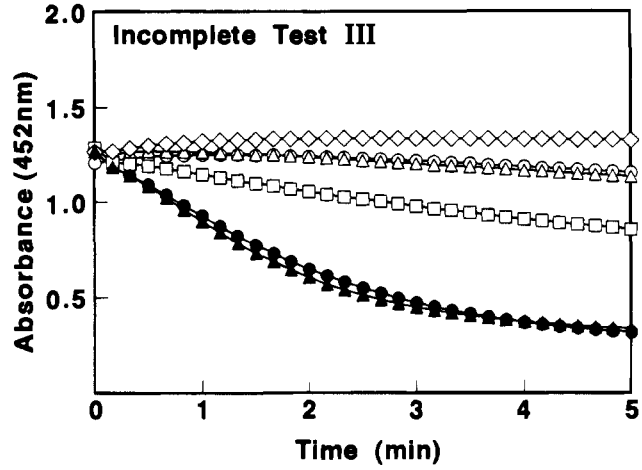

Figure 5. Response of extracts obtained from two normal soybean and four lipoxygenase-lacking cultivars to incomplete test III. Soybean extract (10 μL) obtained from $L_{123(S)}$ (●), $L_{123(F)}$ (▲), L_1 (○), L_2 (△), L_3 (□), and L_0 (◇) was added to the incomplete test III reaction system.

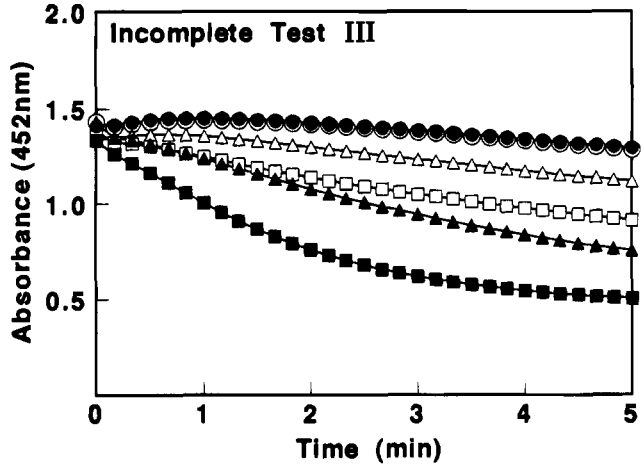

Figure 6. Synergistic effect of L_1 plus L_3 soybean extracts or L_2 plus L_3 soybean extracts. Each mixture of soybean extracts was added to the incomplete test III reaction system: ○, L_1 (20 μL); △, L_2 (20 μL); □, L_3 (20 μL); ●, L_1 (10 μL) plus L_2 (10 μL); ▲, L_1 (10 μL) plus L_3 (10 μL); ■, L_2 (10 μL) plus L_3 (10 μL).

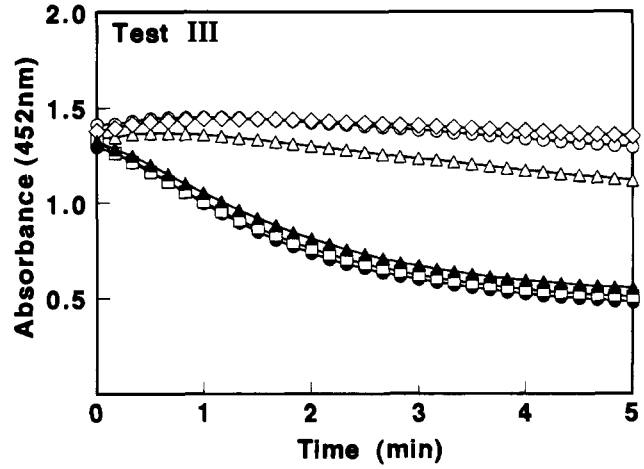

Figure 7. Response of two normal soybean and four lipoxygenase-lacking cultivars to test III. Each soybean extract (10 μL) obtained from $L_{123(S)}$ (●), $L_{123(F)}$ (▲), L1 (○), L_2 (△), L_3 (□), and L_0 (◇) was added to the test III reaction system.

others did not. This result indicated that test II could selectively detect the L-2 isozyme contained in soybean extracts.

Test III for the Detection of the L-3 Isozyme. Carotene bleaching activities of various soybean extracts were examined by incomplete test III. Strong carotene bleaching activity was observed in the reaction mixture with the $L_{123(S)}$ and $L_{123(F)}$ soybean extracts, moderate bleaching with the L_3 soybean extract, and weak bleaching with the L_1, L_2, and L_0 soybean extracts (Figure 5). Because the bleaching activity of the L_3 soybean extract was still too weak for application of a visual judging method, we tried to develop a more sensitive test for detecting the L-3 isozyme.

Ramadoss et al. (1978) demonstrated that combination of either L-1 and L-3 or L-2 and L-3 had a synergistic effect on carotene bleaching. In agreement with their results, we also observed that the L_1, L_2, and L_3 soybean extracts individually exhibited poor carotene bleaching activity; however, the L_3 soybean extract in combination with either the L_1 or L_2 soybean extract showed promoting activity (Figure 6). The combination of L_1 plus L_2 soybean extracts was ineffective. Supporting this evidence, we conducted test III, which was carried out in the presence of the L_2 soybean extract. The L_2 soybean extract was selected because a combination of L_2 plus L_3 soybean extracts exhibited a strong carotene bleaching activity, and the optimum pH for L-2 was near that for L-3.

Figure 7 shows a typical response of various soybean extracts to test III. Three soybean extracts containing the L-3 isozyme ($L_{123(S)}$, $L_{123(F)}$, and L_3 soybean extracts) bleached β-carotene, and their declines in absorbance at 452 nm were clearly distinguishable from that of other soybean extracts lacking the L-3 isozyme. This result indicated that test III could selectively detect the L-3 isozyme contained in soybean extracts.

Visual Judging Method. The limiting factors in applying spectrophotometric method to large-scale screening are the preparation of the soybean extract used as the sample and the use of complex equipment, such as a spectrophotometer. Therefore, we improved the method to make it available as a routine screening test. The procedures, characterized by the use of soybean flour as the sample, are described in detail under Materials and Methods.

The responses of various lipoxygenase-lacking cultivars to tests I–III in visual judging method are shown

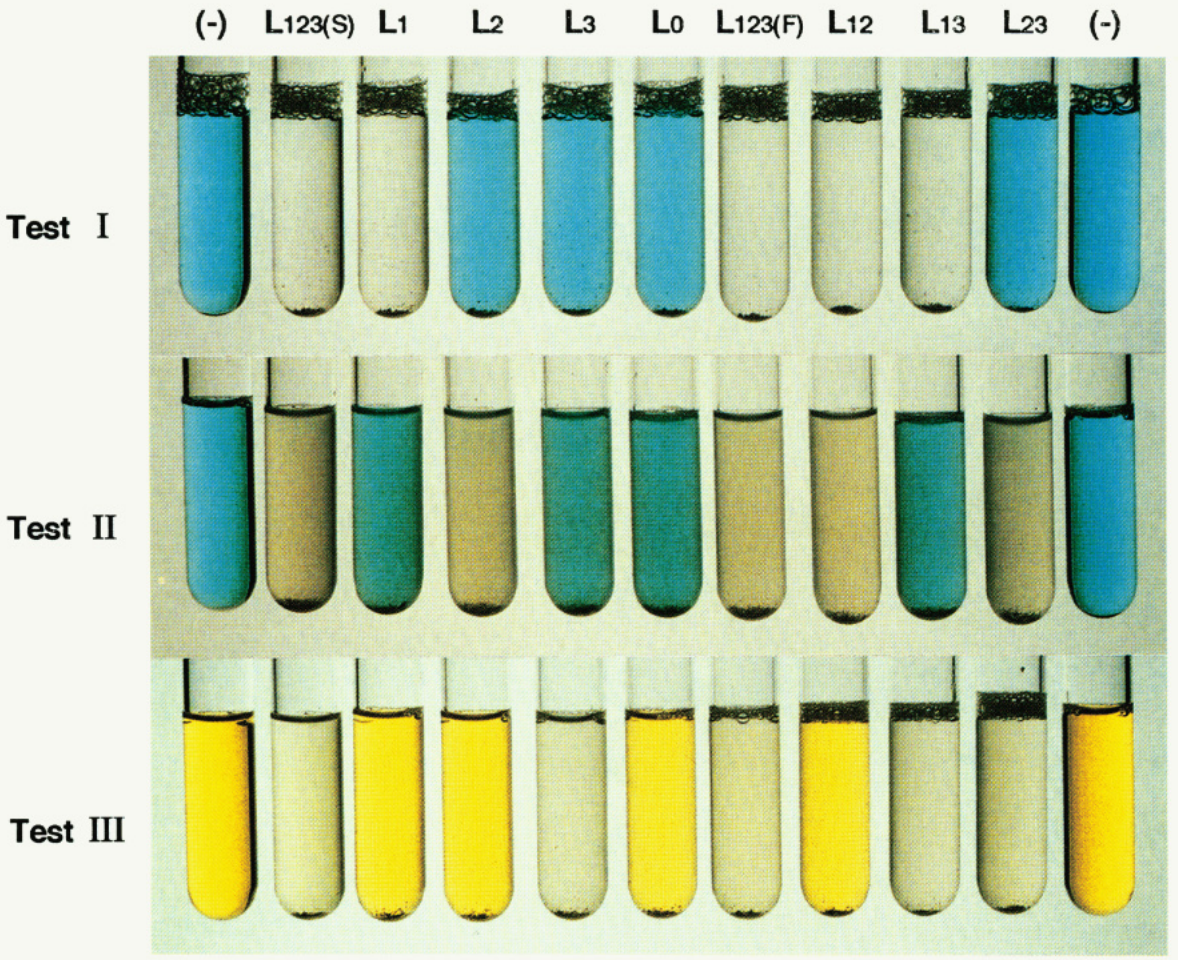

Figure 8. Response of two normal soybean and seven lipoxygenase-lacking cultivars to tests I–III in visual judging method. The soybeans used were abbreviated on the basis of the type of isozymes remaining in the seeds. From the left: no soybean flour, $L_{123(S)}$, L_1, L_2, L_3, L_0, $L_{123(F)}$, L_{12}, L_{13}, L_{23}, and no soybean flour. From the top: test I (3 min with 2.5 mg of soybean flour), test II (5 min with 5.0 mg of soybean flour), and test III (3 min with 2.5 mg of soybean flour).

in Figure 8. Soybeans with strong bleaching activity were $L_{123(S)}$, L_1, $L_{123(F)}$, L_{12}, and L_{13} for test I, $L_{123(S)}$, L_2, $L_{123(F)}$, L_{12}, and L_{23} for test II, and $L_{123(S)}$, L_3, $L_{123(F)}$, L_{13}, and L_{23} for test III, respectively. Thus, tests I, II, or III in visual judging method can specifically identify soybeans containing L-1, L-2, or L-3 isozyme, respectively.

The visual judging method developed here has several advantages. The first advantage is that our method consists of extremely simple procedures. Namely, users have only to add dye–substrate for each test to the wetted soybean flour, whereupon the presence or absence of lipoxygenase isozymes is visualized within 5 min. To design simpler procedures, we used sodium linoleate alone as the substrate in all tests. Also, the reagents required for the tests were less costly. In the cases of other methods (Kikuchi and Kitamura, 1987; Ecochard et al., 1990), two substrates, sodium linoleate and sodium arachidonate, must be prepared. Another advantage is high sensitivity. The total amount of soybean flour required for the three tests is only about 10 mg. Thus, preparation of soybean flour from one soybean seed by drilling into the cotyledons without injury to the hypocotyl will permit its planting after determination. Further, the amount of soybean flour need not be weighed exactly, because the results obtained by each test were not influenced even if the amount of soybean flour used was 4 times that of the standard procedure. The flour held by a small spatula weighs about 5 mg.

Our method is simple, rapid, selective, sensitive, and inexpensive; thus, it can be utilized for large-scale screening. We expect that several trials using this convenient method will be started to obtain lipoxygenase-lacking cultivars with superior agricultural traits such as plant growth, productivity, seed viability, and pathogen pest resistances, especially by breeding of L_{123} and L_0.

ACKNOWLEDGMENT

We are grateful to Dr. K. Kitamura (National Agriculture Research Center, Japan) for the gift of soybean cultivars Yumeyutaka and Kanto 102.

LITERATURE CITED

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

Davies, C. S.; Nielsen, N. C. Genetic analysis of a null-allele for lipoxygenase-2 in soybean. *Crop Sci.* **1986**, *26*, 460–463.

Ecochard, R.; Maltese, S.; Dayde, J.; Planchon, C. On the detection of lipoxygenase iso-enzymes in soybean seeds (*Glycine max*. L. Merrill): use of a simple test for breeders purpose (in French with English summary). *Oleagineux* **1990**, *45*, 333–336.

Hajika, M.; Igita, K.; Kitamura, K. A line lacking all the seed lipoxygenase isozymes in soybean [*Glycine max* (L.) Merrill)] induced by gamma-ray irradiation. *Jpn. J. Breed.* **1991**, *41*, 507–509.

Hajika, M.; Kitamura, K.; Igita, K.; Nakazawa, Y. Genetic relationships among the genes for lipoxygenase-1, -2 and

-3 isozymes in soybean [*Glycine max* (L.) Merrill)] seed. *Jpn. J. Breed.* **1992**, *42*, 787–792.

Hammond, E. G.; Duvick, D. N.; Fehr, W. R.; Hildebrand, D. F.; Lacefield, E. C.; Pfeiffer, T. W. Rapid screening techniques for lipoxygenases in soybean seeds. *Crop Sci.* **1992**, *32*, 820–821.

Hildebrand, D. F.; Hymowitz, T. Two soybean genotypes lacking lipoxygenase-1. *J. Am. Oil Chem. Soc.* **1981**, *58*, 583–586.

Hildebrand, D. F.; Hymowitz, T. Inheritance of lipoxygenase-1 activity in soybean seeds. *Crop Sci.* **1982**, *22*, 851–853.

Kikuchi, A.; Kitamura, K. Simple and rapid carotene bleaching tests for the detection of lipoxygenase isozymes in soybean seeds. *Jpn. J. Breed.* **1987**, *37*, 10–16.

Kitamura, K. Biochemical characterization of lipoxygenase lacking mutants, L-1-less, L-2-less, and L-3-less soybeans. *Agric. Biol. Chem.* **1984**, *48*, 2339–2346.

Kitamura, K. Spontaneous and induced mutations of seed proteins in soybean (*Glycine max* L. Merrill). *Gamma Field Symp.* **1991**, *30*, 61–69.

Kitamura, K.; Davies, C. S.; Kaizuma, N.; Nielsen, N. C. Genetic analysis of a null-allele for lipoxygenase-3 in soybean seeds. *Crop Sci.* **1983**, *23*, 924–927.

Kitamura, K.; Kumagai, T.; Kikuchi, A. Inheritance of lipoxygenase-2 and genetic relationships among genes for lipoxygenase-1, -2 and -3 isozymes in soybean seeds. *Jpn. J. Breed.* **1985**, *35*, 413–420.

Kitamura, K.; Ishimoto, M.; Kikuchi, A.; Kaizuma, N. A new soybean variety "Yumeyutaka" lacking both L-2 and L-3 of the lipoxygenase isozymes (in Japanese with English summary). *Jpn. J. Breed.* **1992**, *42*, 905–913.

Mack, A. J.; Peterman, T. K.; Siedow, J. N. Lipoxygenase isozymes in higher plants: biochemical properties and physiological role. *Isozymes: Curr. Top. Biol. Med. Res.* **1987**, *13*, 127–154.

Nishiba, Y.; Furuta, S.; Hajika, M.; Igita, K.; Suda, I. Hexanal accumulation and DETBA value in homogenate of soybean seeds lacking two or three lipoxygenase isozymes. *J. Agric. Food Chem.* **1995**, *43*, 738–741.

Omura Y.; Akiyama, Y.; Saio, K. Distribution and varietal differences of lipoxygenase in soybean seeds (in Japanese with English summary). *Nippon Shokuhin Kogyo Gakkaishi* **1986**, *33*, 653–658.

Rackis, J. J.; Sessa, D. J.; Honig, D. H. Flavor problems of vegetable food proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 262–271.

Ramadoss, C. S.; Pistorius, E. K.; Axelrod, B. Coupled oxidation of carotene by lipoxygenase requires two isoenzymes. *Arch. Biochem. Biophys.* **1978**, *190*, 549–552.

Toyosaki, T. Bleaching of methylene blue as an index of lipoxygenase activity. *J. AOAC Int.* **1992**, *75*, 1124–1126.

Wang, J.; Fujimoto, K.; Miyazawa, T.; Endo, Y.; Kitamura, K. Sensitivities of lipoxygenase-lacking soybean seeds to accelerated aging and their chemiluminescence levels. *Phytochemistry* **1990**, *29*, 3739–3742.

Wolf, W. J. Lipoxygenase and flavor of soybean protein products. *J. Agric. Food Chem.* **1975**, *23*, 136–141.

Yabuuchi, S.; Lister, R. M.; Axelrod, B.; Wilcox, J. R.; Nielsen, N. C. Enzyme-linked immunosorbent assay for the determination of lipoxygenase isoenzymes in soybean. *Crop Sci.* **1982**, *22*, 333–337.

Received for review July 11, 1994. Revised manuscript received November 17, 1994. Accepted December 30, 1994.[⊗] This study was supported by Grant-in-Aid of Research Program "Development of Methods for Enhancing the Production of Upland Crops with High Quality in Rotational Paddy Fields" provided by the Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan.

JF940376U

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1995.