Degradation of Vitamin E, Vitamin C, and Lutein in Soybean Homogenate: A Comparison of Normal Soybean and Lipoxygenase-Lacking (Triple-Null) Soybean

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The degradation of antioxidative compounds such as vitamin E, vitamin C, and lutein in aqueous homogenate prepared from normal soybean seeds (L_{123}) or lipoxygenase-lacking soybean seeds (triple-null mutant, L_0) was investigated. In the normal soybean, these natural antioxidants decreased rapidly after homogenization. On the contrary, in the L_0 soybean, vitamin E was hardly degraded, and vitamin C and lutein were slowly degraded in comparison with normal soybean. The radical [1,1-diphenyl-2-picrylhydrazyl (DPPH)] scavenging activity of each soybean was determined using an ESR spectrometer, and it was shown that the scavenging activity of normal soybean was weakened after homogenization with water, whereas this reduction was not observed in L_0 soybean. These results suggested that lipoxygenase-lacking soybean could become a superior food ingredient which improves not only the flavor of soybean products but also its nutritional quality and functionality.

Keywords: *Soybean; lipoxygenase; vitamin E; radical scavenging activity; Glycine max*

INTRODUCTION

There is increasing evidence suggesting that radical-mediated reaction is involved in a great variety of diseases, and natural antioxidants in food products possess protective functionality against oxidative damages (Stocker and Frei, 1991; Ramarathnam et al., 1995). Plant materials are rich in antioxidative compounds, such as vitamin E, vitamin C, or carotenoids etc., and are expected to be good ingredients for food products with high antioxidative activity. However, there are some problems during food processing, for example, the degradation of these antioxidants caused by heat treatment or some enzyme reaction (Piironen et al., 1987; Howard et al., 1994; Park et al., 1994). Lipoxygenase, the enzyme catalyzing peroxidation of polyunsaturated fatty acids such as linoleic or linolenic acid, is known to oxidize these antioxidants by its co-oxidation reaction (Eskin et al., 1977; Klein et al., 1985; Håkansson and Jägerstad, 1990). Therefore, a food ingredient with high-lipoxygenase activity can be considered to induce the reduction of the natural antioxidant in food products.

Soybean is an important food ingredient, because of its low cost and high nutrition quality. However, it is also one of the strongest sources of lipoxygenase. Normal soybean seed contains three lipoxygenase isozymes called L-1, L-2, and L-3, respectively (Axelrod et al., 1981), and soybean lipoxygenases are responsible for the generation of undesirable grassy−beany flavors which limit wide utilization of soybeans in food products. Major contributors to these flavors are the volatile carbonyl compounds such as *n*-hexanal, which are the degradation products of lipid hydroperoxides (Rackis et al., 1979; Matoba et al., 1985a,b; Hildebrand, 1989). Recently, Hajika et al. (1991) succeeded in inducing the soybean which lacks all three lipoxygenase isozymes by γ-ray irradiation. In the previous paper, we reported that this triple-null soybean had the lowest levels of both parameters of lipid peroxidation and hexanal accumulation among two or all three lipoxygenase-lacking soybeans, and it was superior as a food ingredient in the improvement of soybean products' flavor (Nishiba et al., 1995).

In the present study, we attempted to clarify that antioxidative compounds were degraded when normal soybean was mixed with water, and this nutritional damage was reduced when lipoxygenase-lacking soybean was used. For this purpose, we determined the changes in natural antioxidant levels in soybean homogenates (endogenous tocopherol and lutein, exogenous ascorbic acid) using wild-type soybean and soybean lacking all three lipoxygenase isozymes. Furthermore, the total radical scavenging activities of aqueous extracts from wild-type or triple-null soybean are compared.

MATERIALS AND METHODS

Soybean Cultivars. Soybean [*Glycine max* (L.) Merr.] cultivars used in this study were as follows: "Suzuyutaka" (L_{123}) was a normal soybean cultivar containing all three types of lipoxygenase isozymes in the seeds. "Ichihime" (L_0, previously called Kyusyu 111) was a cultivar lacking all three types of lipoxygenase isozymes, and it was a derivative of "Suzuyutaka". All seeds were harvested at Kyushu National Agricultural Experiment Station in 1994−1996.

Determination of Tocopherol. Soybean flour was prepared by grinding the seeds with an experimental blender. A suspension of 0.5 g of flour in 9.5 mL of ice-cooled distilled water was homogenized with a Polytron homogenizer (Kinematica, Switzerland) under cooling in an ice bath. The homogenate was kept at 30 °C in a 50 mL centrifugation tube without a cap, and after an appropriate time, 10 mL of 3%

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pyrogallol in ethanol was added to the entire homogenate to stop the enzyme reaction. The saponification was carried out by adding 4 mL of 60% KOH and heating at 70 °C for 30 min with a screw-cap. After ice-cooling, the sample mixture was supplemented with 10 mL of 2.25% NaCl and extracted 6 times with 10 mL of ethyl acetate/hexane (2:8). These extracts were combined, washed twice with water, and then evaporated to dryness under reduced pressure at 40 °C. The resulting residue was dissolved in 1 mL of hexane for the HPLC analysis. The HPLC equipment was JASCO 880-PU with a UV/VIS detector (JASCO 875-UV). HPLC analysis was performed under the following conditions: column, LiChrosorb-NH_2 (5 μm particle size, 250 × 4 mm i.d.; GL Sciences, Inc., Tokyo, Japan); mobile phase, hexane/isopropyl alcohol (98:2, by volume); flow rate, 1.0 mL/min; temperature, 40 °C; detection, 300 nm. Standard vitamin E homologues (for biochemistry and analysis) were purchased from Eizai Co., Ltd., Tokyo, Japan. For the measurement of the initial tocopherol level, soybean flour was added directly to a mixture of 10 mL of 3% pyrogallol in ethanol and 9.5 mL of distilled water and homogenized.

Determination of Lutein. The content of lutein was determined using the same sample that was used for the HPLC analysis of tocopherol. The sample dissolved in hexane was evaporated to dryness, and the residue was dissolved in an equal volume of ethanol for the HPLC analysis of lutein. The HPLC equipment was a JASCO PRETOP 2 system with a mulch channel detector (JASCO MD910), and the conditions were as follows: column, Finepak SIL C18T-5 (5 μm particle size, 250 × 4.6 mm i.d.; JASCO, Tokyo, Japan); mobile phase, methanol; flow rate, 1.0 mL/min; temperature, 40 °C; detection, 444 nm. Standard lutein (xanthophyll) was purchased from Sigma Chemical Co. (Sigma X-6250).

Determination of Ascorbic Acid. A suspension of 0.15 g of soybean flour in 2.85 mL of ice-cooled distilled water containing ascorbic acid (100 μg/mL) was homogenized with a Polytron homogenizer under cooling in an ice bath. The homogenate was kept at 30 °C in a 10 mL test tube without a cap, and after an appropriate time, 3 mL of 10% metaphosphoric acid was added to the entire homogenate to stop the enzyme reaction. The supernatant obtained by centrifugation (12 000 rpm, 10 min, 4 °C, MRX-152, Tomy Seiko Co., Ltd., Tokyo, Japan) was applied to HPLC analysis. The HPLC equipment was the same system as that in the lutein analysis, and the conditions were as follows: column, Inertsil ODS-2 (5 μm particle size, 250 × 4.6 mm i.d.; GL Sciences, Inc.); mobile phase, 0.2% metaphosphoric acid; flow rate, 1.0 mL/min; temperature, room temperature; detection, 243 nm. For the measurement of the initial ascorbic acid level, soybean flour was added to 2.85 mL of 10% metaphosphoric acid containing ascorbic acid at the same concentration and homogenized. To investigate the decrease in ascorbic acid without soybean homogenate, 3 mL of ascorbic acid solution (100 μg/mL) was incubated under the same conditions, and the absorbance (258 nm) was measured by a spectrophotometer (DU-70, Beckman Instruments, Inc.).

Measurement of Radical Scavenging Activity by ESR. A suspension of 0.8 g of soybean flour in 8 mL of ice-cooled distilled water was homogenized with a Polytron homogenizer under cooling in an ice bath. The homogenate was kept at 30 °C for 30 min, and at 95 °C for 10 min to inactivate the enzymes in the homogenate (Omura and Takechi, 1990). The supernatant obtained by centrifugation (11 000 rpm, 10 min, 4 °C) was used for the sample for measurement of radical scavenging activity by ESR spectrometry. In determining the initial radical scavenging activity, soybean flour was added to 8 mL of 95 °C distilled water; it was kept at 95 °C for 10 min and homogenized. In this study, the scavenging activity toward 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined. The DPPH radical was analyzed as follows: The sample extract (x μL) was added to a mixture of 100 μM DPPH in 50% ethanol (100 μL), ethanol (x μL), and 50% ethanol (200−2x μL). This was then mixed vigorously for 5 s, transferred to an aqueous quartz flat cell (JEOL LC-11 cuvette), and placed into the cavity of the ESR spectrometer (JES-RE1X, JEOL Co., Ltd., Tokyo, Japan). Recording of the ESR spectra started exactly 60 s after the addition of the sample. ESR spectra were recorded at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.25 mT; scanning field, 335.8 ± 5 mT; receiver gain, 250; response time, 0.1 s; sweep time, 2.0 min; microwave power, 8 mW; and microwave frequency, 9.450 GHz.

Table 1. Initial Levels of Tocopherol and Lutein Content in "Suzuyutaka" (L_{123}) and "Ichihime" (L_0)[a]

	μg/g of soybean flour	
	Suzuyutaka	Ichihime
α-Toc	36.18 ± 1.02	32.24 ± 1.70
β-Toc	11.71 ± 0.22	9.48 ± 0.89
γ-Toc	178.35 ± 4.07	190.51 ± 6.32
δ-Toc	89.14 ± 4.84	91.88 ± 6.74
lutein	2.58 ± 0.10	3.32 ± 0.25

[a] Results are expressed as means ± standard deviations of four experiments.

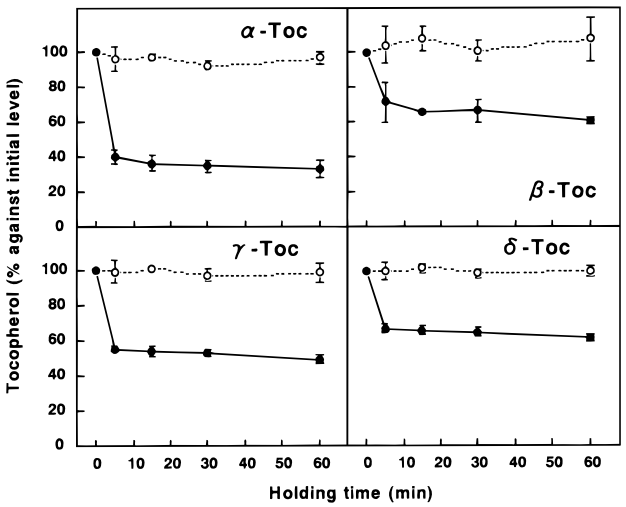

Figure 1. Changes in tocopherol content in soybean homogenates. Soybean flour was homogenized with distilled water and kept at 30 °C. After an appropriate time, the remaining tocopherol content was determined. Results are expressed as means ± standard deviations of four experiments. (●) "Suzuyutaka" (L_{123}); (○) "Ichihime" (L_0).

RESULTS

Changes in Tocopherol Content in Soybean Homogenates. Table 1 shows the initial levels of tocopherol and lutein content in normal soybean seeds (Suzuyutaka, L_{123}) and triple-null soybean seeds (Ichihime, L_0). Both soybean seeds had almost the same levels of tocopherol and lutein. The homogenates of L_{123} and L_0 soybean seeds were then prepared from soybean flour, and the retention of tocopherol content in the homogenate was determined as a function of holding time at 30 °C after homogenization (Figure 1). In the L_{123} soybean homogenate, tocopherol levels rapidly decreased during the first 5 min after which time only a slight change in the retention was observed. Among tocopherol homologues, the α-tocopherol decrease was most intensive, and its retention reached about 40% in a few minutes. On the contrary, their decrease was not observed in the L_0 soybean homogenate.

Changes in Lutein Content in Soybean Homogenates. The time course study showed rapid reduction at the initial stage and subsequent slowing in lutein content in the L_{123} soybean flour homogenate

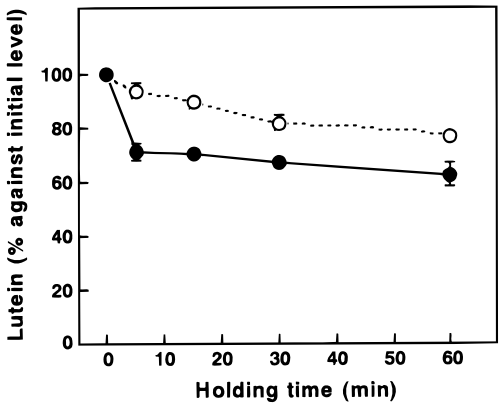

Figure 2. Changes in lutein content in soybean homogenates. Soybean flour was homogenized with distilled water and kept at 30 °C. After an appropriate time, the remaining lutein content was determined. Results are expressed as means ± standard deviations of four experiments. (●) "Suzuyutaka" (L_{123}); (○) "Ichihime" (L_0).

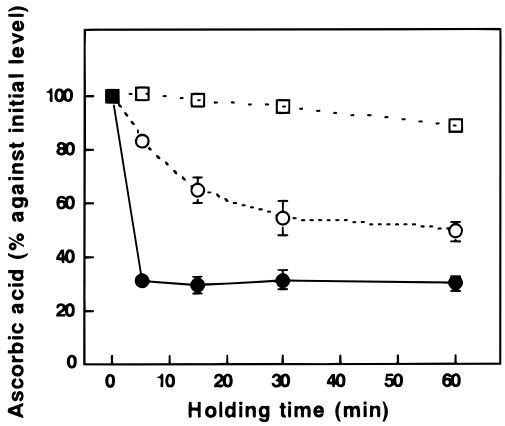

Figure 3. Changes in ascorbic acid content in soybean homogenates. Soybean flour was homogenized with distilled water containing ascorbic acid (100 μg/mL) and kept at 30 °C. After an appropriate time, the remaining ascorbic acid content was determined. Results are expressed as means ± standard deviations of four experiments for the soybean homogenates, and the result of a single experiment for the ascorbic acid solution without soybean homogenate. (●) "Suzuyutaka" (L_{123}); (○) "Ichihime" (L_0); (□) without soybean homogenate.

(Figure 2). In the L_0 soybean homogenate, the lutein contentdecreased slowly throughout the holding time.

Changes in Ascorbic Acid Content in Soybean Homogenates. Because ascorbic acid was not detected in soybean seeds, the effect of soybean homogenate against ascorbic acid degradation was investigated by the addition of exogenous ascorbic acid (1.9 mg/g of soybean seed, Figure 3). In the homogenate of the L_{123} soybean, ascorbic acid rapidly decreased, and its retention reached about 30% within the first 5 min. On the other hand, the ascorbic acid content gradually decreased in the L_0 soybean homogenate; however, its reduction rate was slower than that of the L_{123} soybean.

Comparison of the Total Radical Scavenging Activity in Aqueous Extracts of L_{123} or L_0 Soybean Seeds. A typical ESR spectrum of DPPH and dose−response curves for the radical (DPPH) scavenging activity of soybean aqueous extracts are shown in Figures 4 and 5. As for extracts with heat treatment before incubation at 30 °C, the radical scavenging activity was similar between L_{123} and L_0 soybean. The IC_{50} values (μL/200 μL assay) of each extract were 6.87 ± 0.058 (L_{123}) and 6.53 ± 0.493 (L_0). On the other hand,

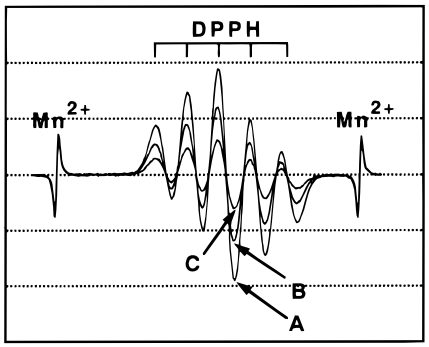

Figure 4. Effect of soybean aqueous extract on the ESR spectrum of DPPH. Soybean homogenate was kept at 30 °C for 30 min and heated at 95 °C for 10 min to inactivate the enzymes in the homogenate. The DPPH radical scavenging activity of the supernatant of the homogenate was measured. A, Control (no addition); B, "Suzuyutaka" (10 μL/assay); C, "Ichihime" (10 μL/assay).

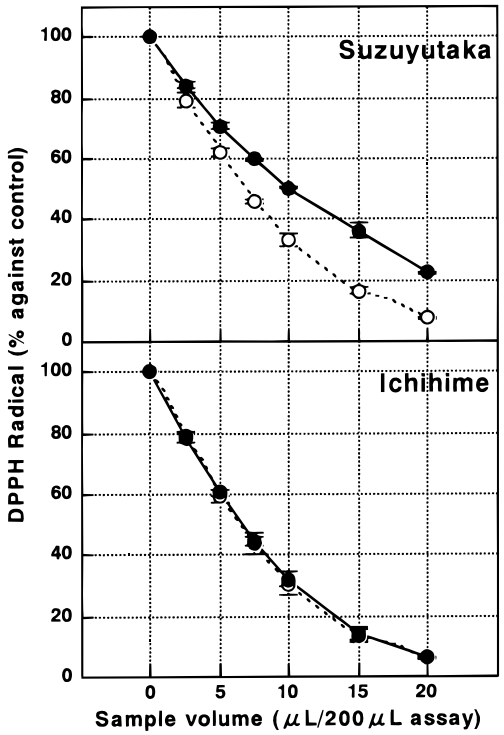

Figure 5. Dose−response curves for the radical (DPPH) scavenging activity of soybean aqueous extracts. Soybean homogenate was kept at 30 °C for 30 min and heated at 95 °C for 10 min to inactivate the enzymes. The supernatant of the homogenates was used for measurement of radical scavenging activity by an ESR spectrometer. In the case of initial radical scavenging activity, soybean flour was added to 95 °C distilled water and kept at 95 °C for 10 min and homogenized. Results are expressed as means ± standard deviations of three experiments. (●) Radical scavenging activity after incubating at 30 °C for 30 min; (○) initial radical scavenging activity.

after 30 min incubation at 30 °C, the radical scavenging activity of the L_{123} soybean extract was weakened (IC_{50} 10.17 ± 0.153), whereas no reduction was observed in the extract of the L_0 soybean (IC_{50} 6.67 ± 0.115).

DISCUSSION

The present study clearly demonstrated that there were significant differences in the changes in the natural antioxidants between L_{123} and L_0 soybean after homogenization with water (Table 1, Figures 1 and 2),

although the initial levels were similar. In the process of lipoxygenase reaction, peroxyradicals are formed from polyunsaturated fatty acids and oxygen, and it has been reported that a considerable proportion of peroxyradicals was not directly converted to hydroperoxides (Weber and Grosch, 1976). Therefore, it is possible that these free radicals attack the other easily oxidizable element in foodstuff and cause a decline in the nutritional value. In this study, natural radical scavengers contained in soybean seeds were consumed during the incubation of the homogenate, and the total radical scavenging activity was weakened in the L_{123} soybean (Figures 4 and 5). In addition to the molecular species examined in this study, chlorophyll, cytochrome *c*, thiols, etc. were reported to suffer co-oxidation by lipoxygenases (Eskin et al., 1977).

In the previous study, we investigated the time course of lipid peroxidation (DETBA value) and hexanal accumulation using the same cultivars, and demonstrated that lipid peroxidation and hexanal accumulation of wild-type soybean homogenate rose rapidly and intensely for a few minutes after homogenization (Nishiba et al., 1995), and their suppression seemed to be difficult. Similarly, all of the antioxidative compounds investigated in this study decreased rapidly during the first 5 min in the L_{123} soybean homogenate. Thus, the reduction rate of these antioxidative compounds was ranked with the rate of lipid peroxidation. In the actual food processing, the extent of this nutritional damage will vary according to the following factors: ratio of soybean in food products, processing temperature, polyunsaturated fatty acids contained in other ingredients, the condition of air supply, and so on. Judging from our results, the effect of soybean lipoxygenases on the nutrients which are sensitive to oxidation is considerable, and the control of this nutrient damage seems to be difficult as is that of the generation of off-flavor originating from lipoxygenase reaction. However, if L_0 soybean is used, these losses are not observed or improved considerably; therefore, it will become easy to maintain these natural antioxidants.

In a manufacturing process of food products, either the enzymatic or the nonenzymatic reactions may be concerned with the loss of antioxidative nutrients, and the proportion of enzymatic reaction to whole nutrient damage seems to depend on the food ingredients or the manufacturing conditions. In this study, the reduction of tocopherols was not observed in the L_0 soybean homogenate (Figure 1). Therefore, in soybean aqueous homogenates near room temperature, lipoxygenases are major contributors reducing the vitamin E content. Among tocopherol homologues, α-tocopherol was the most unstable in the L_{123} homogenate (Figure 1), though it is the most important homologue because of its high physiological activity. It is known that α-tocopherol has the highest scavenging activity to free radicals (Niki et al., 1986; Mukai et al., 1986); therefore, it seems to be consumed most rapidly in soybean seeds when mixed with water.

The content of ascorbic acid (added exogenously) or lutein rapidly decreased in the L_{123} homogenate, and it also gradually decreased in the L_0 soybean (Figures 2 and 3). This means that there are other enzymes such as oxidase to reduce these molecules, or factors to cause nonenzymatic degradation. However, judging from the L_{123} time course, their effect appears to be smaller than the effect of lipoxygenase activities.

In this study, we demonstrated that antioxidative compounds such as vitamin E were unstable in normal soybean aqueous homogenate, and the greater part of these nutrients could be lost within a few minutes depending on the manufacturing conditions. On the other hand, in the L_0 soybean homogenate, the loss of these antioxidants was remarkably reduced. Therefore, when food producers use soybean in their food products and want to minimize the loss of natural antioxidants or easily oxidizable nutrients, lipoxygenase-lacking soybeans appear superior as a food ingredient from the standpoints of food nutrition and functionality, in addition to the improvement of the soybean product's flavor.

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