Oxidative Stability of Bran Lipids from Rice Variety [*Oryza sativa* (L.)] Lacking Lipoxygenase-3 in Seeds

Yasuhiro Suzuki,*,† Takeshi Yasui,† Ushio Matsukura,† and Junji Terao‡

National Agriculture Research Center and National Food Research Institute, Kannondai, Tsukuba, Ibaraki 305, Japan

Lipoxygenase-3 (LOX-3) is the major component among isozymes in rice seeds. Using the Thai rice variety Daw Dam, which lacks LOX-3 in its seed, and the Japanese rice varieties Koshihikari and Koganemochi, which have normal LOX-3 activity, the oxidative stability of lipids in rice bran fractions during storage at 4 and 37 °C was studied. Free fatty acid content in lipids differed between storage temperatures and peroxide value (POV) differed among varieties. Differences among varieties and between storage temperatures in carbonyl value (COV) in lipids were observed. The amounts of free linoleic and linolenic acids did not differ among varieties during storage at either temperature. POV and COV in Daw Dam were lower, however, than in the other two varieties. These results suggest that the peroxidation of unsaturated fatty acids occurs at lower levels in the Daw Dam bran fraction than in the varieties with LOX-3 in their seed. The effect of this lack of LOX-3 as it relates to rice grain stability is discussed.

Keywords: *Bran lipid; carbonyl compound; free fatty acid; lipoxygenase-3; peroxide; rice*

INTRODUCTION

Rice, the staple food cereal in East and Southeast Asia, is used as a supplementary food in many countries. Japan is self-sufficient in rice, but variability of rice supply makes the stability of storage important in ensuring ongoing high quality rice availability. From this viewpoint, rice grain deterioration and the development of a stale flavor during storage are serious problems that reduce the quality of stored rice. Many efforts have been made to stabilize the storage quality of brown rice. Optimum packaging materials, temperatures, and storage atmospheres help slow oxidative deterioration (Mitsuda et al., 1972; Sowbhagya and Bhattacharya, 1976; Ory et al., 1980; Sharp and Timme, 1986). The high cost of land and construction, however, complicate the building of temperature-controlled warehouses that would help ensure the success of these postharvest rice treatments.

Many investigators have reported that free fatty acid (FFA) content increases during storage and suggest that the degradation of lipids is responsible for the deteriorative changes during storage (Yasumatsu and Moritaka, 1964; Yasumatsu et al., 1966; Aibara et al., 1986; Shin et al., 1986; Takano, 1993). Lipoxygenase (LOX) catalyzes the oxidation of polyunsaturated fatty acids containing a 1,4-pentadiene structure, such as linoleic and linolenic acids, into conjugated hydroperoxy fatty acids. Hydroperoxides are further transformed into various volatile compounds causing changes in or adding flavor, and LOX is related at least in part to the formation of such volatile compounds (Gardner, 1988, 1995; Hildebrand, 1989; Hatanaka, 1993). Thus, the absence of LOX enzymes in rice grains may reduce oxidative deterioration.

LOX activity in rice grain is localized in the bran milling fraction (Shastry and Rao, 1975; Yamamoto et al., 1980). Three isozymes, LOX-1, LOX-2, and LOX-3, were found in rice embryos, and LOX-3 is the major component of isozymes (Ida et al., 1983). To breed a new rice variety with good storage stability, Suzuki et al. (1992, 1993) screened a rice variety lacking LOX-3 from a germplasm collection with monoclonal antibodies specific to LOX-3 in embryos. A Thai variety, Daw Dam, was recently found, by immunoblot analysis and enzymatic assay for LOX-3 activity (Suzuki et al., 1993), to lack the LOX-3 protein. Based on the results of crossing Daw Dam with rice cultivars with normal LOX-3 activity, Suzuki et al. (1995, 1996) concluded that the absence of LOX-3 is inherited as a single recessive trait. This conclusion means that breeding rice varieties without LOX-3 (LOX-3-less) is possible.

Our objective was to study the stability of bran lipids from the LOX-3-less variety and to compare it with those from varieties with normal LOX-3 activity.

EXPERIMENTAL PROCEDURES

Rice Samples. We used three rice varieties in our experiments: Daw Dam, a Thai variety lacking LOX-3 in its seed and having a glutinous endosperm (Suzuki et al., 1993, 1996); Koshihikari, the most popular nonglutinous variety in Japan; and Koganemochi, the most popular glutinous variety in Japan. Samples were collected from an experimental field managed by National Agriculture Research Center, Japan, in 1993, and stored at 4 °C until the study was initiated, less than a year later. Paddies were equilibrated to room temperature for 1 day, and then were husked. Rice bran for each variety was polished from 300 g of brown rice with an experimental grain polisher (Pearlest, Kett. Company, Tokyo, Japan). Rice bran was divided among polyethylene tubes and stored at 4 or 37 °C for 1 or 2 months.

Extraction of Lipids. Analytical-grade reagents (Nakarai Tesque, Inc., Kyoto, Japan) were used for lipid extraction. A 5-g sample of the rice bran fraction stored for a stated period of time was homogenized with 25 mL of chloroform:methanol (3:2, v/v) solution containing 0.05% butylhydroxytoluene (CM solution) with a polytron (Kinematica, Switzerland). After the homogenate was centrifuged, the pellet was homogenized two times with another 25 mL of CM solution. The extracts were combined and concentrated on a rotary evaporator, and CM

* Author to whom correspondence should be addressed (e-mail suzuyasu@narc.affrc.go.jp).
† National Agriculture Research Center.
‡ National Food Research Institute.

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solution was added to 30 mL volume. The extract was washed three times with distilled water according to the method of Folch et al. (1957). The chloroform layer was concentrated on a rotary evaporator, and the residue was weighed as total lipids. Lipids were dissolved in 10 mL of CM solution and then stored at below −40 °C.

Gas Chromatographic Analysis of Fatty Acids. Lipids were transesterified for 2 h at 80 °C with methanolic HCl containing 0.5 μg of methyl heptadecanoate as an internal standard (Serdary Research Laboratories, Inc.). Fatty acid methylesters were extracted with heptane and analyzed on a gas chromatograph (GC); Hewlett-Packard 5890 series II; Cheadle Heath, U.K.) equipped with a flame-ionization detector and a capillary column (Supelcowax 10; 30 m × 0.53 mm i.d., 1.00 μm film; Supelco, Inc., Bellefonte, PA). The column temperature was programmed from 90 to 220 °C (30 °C/min), holding the initial temperature for 1 min and the final temperature for 10 min. The injection temperature was 280 °C and the detector temperature was 250 °C.

Free Fatty Acid Contents. The amount of FFA in total lipids was determined colorimetrically by the method of Itaya (1977). The FFA content was calculated as oleic acid and expressed as micromole per lipid. FFAs were separated from other lipids by thin-layer chromatography with silica gel G (Uniplate, Analtech, Newark, NJ) and a solvent system consisting of hexane, diethylether, and acetic acid (80:30:1, v/v/v). Spots were detected with iodine vapor. Layers containing FFAs were scraped off, and the obtained FFAs were converted to methyl esters with HCl-methanol, and analyzed by GC, as described to analyze the fatty acid compositions of total lipids. Fatty acids were measured in duplicate and averages were used. Variation of data was negligible.

Measurement of Peroxide and Carbonyl Values. The peroxide value (POV) in total lipids was determined colorimetrically by a cadmium acetate method according to Buege and Aust (1978), and calculated with cumene hydroperoxide as a peroxide standard. Total carbonyl compounds in total lipids were estimated by measuring the carbonyl value (COV) by the method of Kumazawa and Oyama (1965), with *n*-hexanal as a carbonyl standard. POV and COV were measured in duplicate and averages were used. Variation of data was negligible.

Lipoxygenase Content. The production of monoclonal antibodies specific to LOX-3 has been described elsewhere (Suzuki et al., 1992). LOX-3 content was analyzed according to the method of Suzuki et al. (1993). A manually selected rice embryo was homogenized individually with 40 μL of 62.5 mM Tris-HCl (pH 6.8), containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mM monoiodoacetic acid, 0.08% bromophenol blue, and 10% glycerol. SDS-polyacrylamide gel electrophoresis, a Western blot, and measurement of the LOX-3 content were conducted as described previously (Suzuki et al., 1992). LOX activities were analyzed according to the method of Axelrod et al. (1981).

RESULTS

LOX-3 Content. Suzuki et al. (1993) reported that Daw Dam lacked the LOX-3 band on the basis of results of immunoblot analysis and an enzymatic assay for LOX-3 activity. We compared LOX-3 content in the embryos of Daw Dam and two popular varieties in Japan as determined by immunoblot analysis (Figure 1). A LOX-3 band was detected in extracts from the embryos of Koshihikari and Koganemochi, but not in extracts from embryos of Daw Dam.

Content and Composition of Fatty Acids. Fat acidity is one of the important indices in the changes in rice qualities during storage (Takano, 1993). FFA content in all varieties increased with increasing storage periods at both storage temperatures (Figure 2). Contents in all varieties stored at 37 °C were higher than those at 4 °C (i.e., about seven times higher after 2 months than at the time storage started). The FFA content in the Daw Dam variety was slightly higher than or equal to that in varieties with normal LOX-3 activity.

Figure 1. LOX-3 content in seeds from Koshihikari (lane 1), Daw Dam (lane 2), and Koganemochi (lane 3). Western blot analysis of LOX-3 in an embryo of each variety was conducted as described under Experimental Procedures.

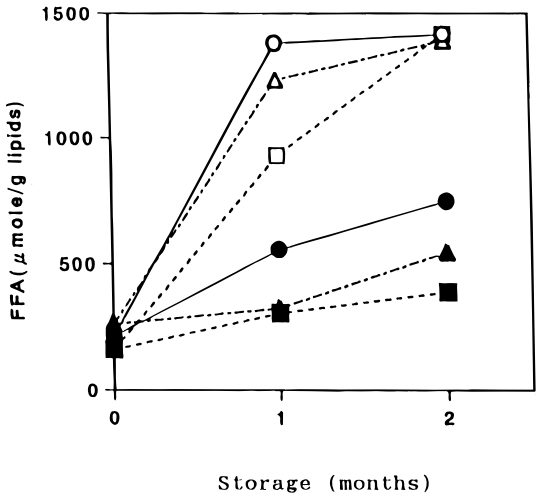

Figure 2. Changes in free fatty acids levels in bran fractions from Daw Dam (●, ○), Koshihikari (■, □), and Koganemochi (▲, △) stored at 4 °C (●, ■, ▲) or at 37 °C (○, □, △).

A comparison of the fatty acid composition of total lipids in the three rice varieties showed that total lipids consisted of 38−42% oleic acid, 37−38% linoleic acid, 15−17% palmitic acid, and small amounts (1−3%) of stearic and linolenic acid (Table 1). These results agreed well with those from other reports (Aibara et al., 1986; Taira et al., 1988). Only minor differences were found in the composition for the three rice varieties.

A comparison of the FFA fraction composition after storage for 1 and 2 months at 4 and 37 °C showed oleic, linoleic, and palmitic acids to be the major FFAs for each variety (Table 2). The proportion of oleic, linoleic, and linolenic acids in samples stored at 37 °C was slightly higher than for samples stored at 4 °C.

Oxidation Products. POVs in total lipids of the three varieties increased during storage (Figure 3). The POV in Koganemochi increased rapidly during the first month of storage and maintained the highest level thereafter. The maximum level of POV was <10 μmol/g

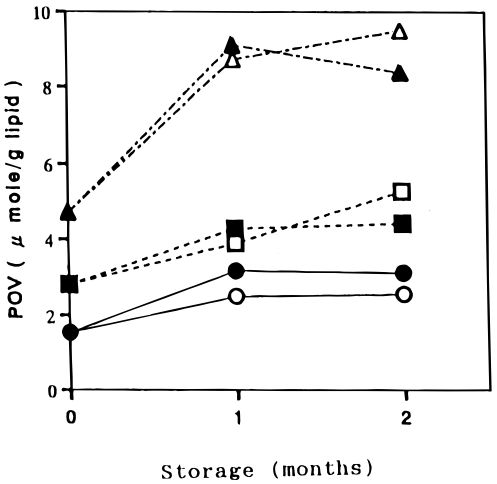

Figure 3. Changes in peroxides levels in bran fractions from Daw Dam (●, ○), Koshihikari (■, □), and Koganemochi (▲, △) stored at 4 °C (●, ■, ▲) or at 37 °C (○, □, △).

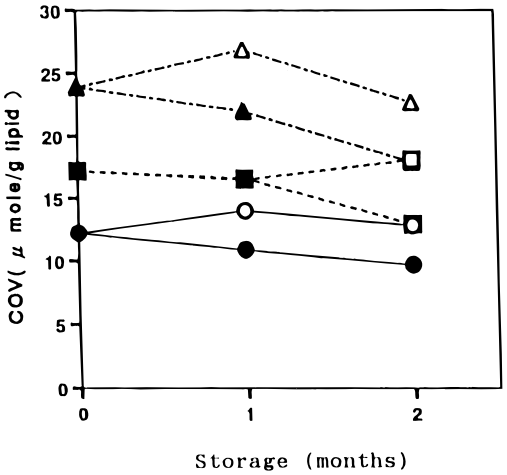

Figure 4. Changes in carbonyl compounds levels in bran fractions from Daw Dam (●, ○), Koshihikari (■, □), and Koganemochi (▲, △) stored at 4 °C (●, ■, ▲) or at 37 °C (○, □, △).

Table 1. Fatty Acid Composition of Bran Fractions from Rice Varieties with LOX-3 in Seeds (Koshihikari and Koganemochi) and without LOX-3 (Daw Dam) at the Beginning of Storage

	fatty acid content (%)		
fatty acid	Daw Dam	Koshihikari	Koganemochi
myristate (14:0)	0.5	0.4	0.6
palmitate (16:0)	15.0	16.6	17.4
palmitoleate (16:1)	0.3	0.3	0.3
stearate (18:0)	1.8	2.3	1.8
oleate (18:1)	41.8	38.0	38.1
linoleate (18:2)	37.3	38.1	37.9
linolenate (18:3)	1.4	1.7	1.5
arachidate (20:0)	0.5	0.7	0.6
eicosenoate (20:1)	0.4	0.5	0.5
behenate (22:0)	0.3	0.4	0.4
erucate (22:1)	0.1	0.1	0.1
lignocerate (24:0)	0.4	0.6	0.5

of total lipids, or 1% of FFA contents. In contrast to the time course in FFA contents, the POVs in all varieties stored at 4 °C were almost the same as those at 37 °C. The POV in Daw Dam was the lowest of all varieties throughout the storage period at both temperatures.

We also analyzed changes in COV during storage (Figure 4). COVs in all varieties at 4 °C were lower than those at 37 °C, with COVs at 4 °C gradually decreasing in proportion to the storage period and those at 37 °C showing almost no change during storage periods. Values for Daw Dam were lower than for other varieties at both storage temperatures.

DISCUSSION

The purpose of our experiments was to determine whether the oxidative stability of lipids in rice bran fractions was related to whether the seed lacked LOX-3. To do so, we compared Daw Dam rice, which lacks LOX-3 activity in its seed, and Koshihikari and Koganemochi rice, which have normal LOX-3 activity (Suzuki et al., 1993; Figure 1). A comparison of FFA contents, POV, and COV in total lipids showed some differences among varieties and also in storage effects at two different temperatures (Figures 2−4; Tables 1 and 2).

Rice bran, potentially a good source of vegetable oil, does not keep as well as other vegetable materials, mainly because of the high lipolytic activity in the rice bran fraction (Takano, 1993). Physical damage during harvesting, transportation, and storage causes direct contact of the enzymes with substrates, and high temperatures accelerate the enzymatic reaction (Takano, 1993; Ohta et al., 1990). Immediately after rice milling is completed, triacylglycerols in rice bran decompose rapidly, releasing FFAs, and, as we showed in this report, large increases in FFA in total lipids from bran fractions occurred at 37 °C in all varieties (Figure 2). Free linoleic and linolenic acid content of total lipids in Daw Dam remained at the same level as other varieties during storage (Figure 2; Table 2). This result indicates that the amount of substrates for LOX-3 in Daw Dam bran was comparable to that in the other varieties.

The FFA fraction stored at 4 °C contained more palmitic and stearic acids and less oleic and linoleic acids than that stored at 37 °C (Table 2). A similar result was also reported by Aoyagi et al. (1985). Yasumatsu and Moritaka (1964) reported the differences of fatty acid compositions of hulled rice and polished rice during storage, and suggested one of the reasons for this difference in fatty acid compositions is the differences in rate of fatty acid liberation.

Table 2. Composition of Free Fatty Acids Stored for 1 or 2 Months[a]

	1 month						2 months					
	Daw Dam		Koshihikari		Koganemochi		Daw Dam		Koshihikari		Koganemochi	
FFA	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
myristate (14:0)	0.7	0.6	0.6	0.6	1.0	0.8	0.6	0.6	0.9	0.4	1.1	0.6
palmitate (16:0)	24.0	17.9	26.1	21.1	29.4	23.0	21.4	12.6	24.8	18.4	28.0	19.5
stearate (18:0)	3.2	2.4	5.3	3.0	4.5	2.2	3.1	1.6	4.8	2.7	3.6	2.1
oleate (18:1)	41.5	45.2	37.6	42.8	33.7	35.5	41.3	45.7	37.7	40.2	32.7	36.8
linoleate (18:2)	27.1	30.5	24.5	28.1	26.4	34.8	29.8	36.6	27.3	34.5	29.4	37.5
linolenate (18:3)	0.9	1.1	0.9	1.2	0.8	1.3	1.0	1.2	0.7	1.5	1.0	1.4

[a] Bran fractions from Daw Dam, Koshihikari, and Koganemochi were analyzed as described under Experimental Procedures.

Linoleic and linolenic acids liberated from triacylglycerols are converted to their hydroperoxides by LOX enzymes (Gardner, 1988, 1995; Hildebrand, 1989). Fatty acid hydroperoxides are degraded to form volatile carbonyl compounds that impart an off-flavor and rancid odor to brown rice (Yasumatsu et al., 1966; Sharp and Timme, 1986; Shin et al., 1986). As shown by the level of POV (Figure 3), hydroperoxides of unsaturated fatty acids were lower in Daw Dam than in Koshihikari and Koganemochi. Carbonyl compounds derived from hydroperoxides were the lowest in Daw Dam, as indicated by the COV (Figure 4). Daw Dam seed lacks LOX-3, as analyzed immunologically, whereas the seeds of the other two varieties contain the LOX-3 protein (Figure 1). Elution profiles of embryo homogenate extracts showed that Koshihikari, but not Daw Dam, gave a main peak of LOX-3 activity on a DEAE-5PW column (Suzuki et al., 1993). These results suggest that the peroxidation of unsaturated fatty acids in the bran fraction occurs at a lower level in Daw Dam than in the other two varieties.

Because the amounts of FFA, POV, and COV have been adopted as indices for determining storage periods, it is important to clarify the relationships among FFA, POV, and COV. Although FFA contents in all varieties stored at 37 °C were higher than those at 4 °C, POVs stored at 37 °C were almost the same as those at 4 °C. In our findings, the amounts of peroxides were ~1% of the FFA (Figures 2 and 3) and COVs were two to three times higher than POVs (Figures 3 and 4). These results suggest that the rate of peroxidation in unsaturated fatty acids is slower than the rate of FFA and carbonyl compound formation, and that hydroperoxides stored at 37 °C decompose faster than those stored at 4 °C. This conclusion is supported by the fact that COVs of varieties stored at 37 °C were high compared with those stored at 4 °C.

The enzymatic and nonenzymatic peroxidation of unsaturated fatty acids should also be taken into account, in addition to LOX-3 catalyzed peroxidation. Although rice bran contains LOX-1 and LOX-2 in addition to LOX-3, LOX-3 is the major rice grain isozyme (Ida et al., 1983; Ohta et al., 1986), and the crude extract and partially purified LOX-3 from embryos showed similar high-performance liquid chromatography profiles for the reaction products (Ida et al., 1983). A similar peroxidation rate can be expected if three varieties have LOX-1 and LOX-2, so it is unlikely that the enzymatic reaction catalyzed by LOX-1 and LOX-2 is a major factor that causes varietal differences in POV and COV. The participation of nonenzymatic peroxidation in varietal differences also appears excluded because it is not likely that nonenzymatic peroxidation in Daw Dam was lower than in other varieties.

Based on data (Suzuki, 1995; Suzuki et al., 1996) for F_1, F_2, and B_1F_1 seeds analyzed by crossing Daw Dam with rice varieties having normal LOX-3 activity, we concluded that LOX-3 is controlled by a single gene and the absence of LOX-3 is recessive. Breeding programs in LOX-less varieties of soybeans are in progress to improve flavor (Kitamura, 1995). To improve rice grain stability, we have been breeding new lines with low levels of peroxidative products through the use of this recessive allele. Because external factors favoring the enzymatic peroxidation of unsaturated fatty acids cannot be easily controlled, the genetic elimination of LOXs from seed would be a useful method for storage to maintain high-quality rice.

ABBREVIATIONS USED

CM solution, chloroform:methanol (3:2, v/v) solution containing 0.05% butylhydroxytoluene; COV, carbonyl value; FFA, free fatty acid; GC, gas chromatography; LOX, lipoxygenase; POV, peroxide value; SDS, sodium dodecyl sulfate.

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