Lipoxygenase Activity in Black Rices

You-Seok Lee, Seon-Joo Song and Chong-Ouk Rhee*

Department of Food Science and Technology, Chonnam National University, Kwangju 500-757, Korea

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Lipoxygenase activities of three cultivars of black rice (Chindo, Suwon-415, Yongkeum-1) were determined using an oxygen polarographic method to measure oxygen uptake. Studies at different pH levels revealed that the optimum pH was about pH 7.0 for Suwon-415 and pH 7.5 for Chindo and Yongkeum-1. The specific activities of Chindo, Suwon-415 and Yongkeum-1 at optimal pH were 41.0, 27.3, and 29.6 unit/mg-protein, respectively. In all the cultivars, there was an increase in the activity with increase in reaction temperature. Enzyme activity was tested at different concentrations of the substrate. The resulting K_m and V_{max} values of Chindo, Suwon-415, and Youngkeum-1 were 0.059, 0.050, and 0.066 mM and 2020, 2283, and 1616 unit/g-grain, respectively. Enzyme activity decreased at all storage temperatures(25, 4, and -40°C).

Key words : *black rice, lipoxygenase, oxygen polarographic method.*

Lipoxygenase(LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase that catalyzes, as a primary reaction, the hydroperoxidation of linoleic acid and other polyunsaturated lipids that contain a *cis,cis,*-1,4-pentadiene moiety by molecular oxygen.[1] Under appropriate conditions this enzyme leads to the deterioration of fat-soluble vitamins and essential fatty acids in oils and fats. It also produces off-flavors and odors due to its action on unsaturated fatty acids in the lipids of food materials.[2]

Aibara *et al.*[3] reported that the free fatty acid contents gradually increased during preservation of rice grain. These fatty acids, particularly linoleic acid, are easily oxidized by LOX, and consequently the oxidation causes deterioration of the quality of rice. Sekhar *et al.*[4] revealed that LOX of dehusked rice had optimal activity around pH 8.0.

LOX-catalyzed lipid oxidation can be analyzed by several methods. One includes observing the increase in absorption at 234 nm arising from the conjugated double bonds formed during the reaction. Another is to measuring the use of oxygen with the aid of a recording oxygen electrode. The latter, polarographic method, is widely used since it is sensitive and rapid. It only requires 3~5 min reaction time, does not require an optically clear solution, and is applicable to both crude and pure enzyme preparations. Ordinary spectrophotometric methods are not suitable since they require optically clear enzyme extracts obtainable only through time consuming procedures.[5] Assays based on O_2 uptake and employing O_2 electrode are useful especially for rough monitoring of the LOX reaction.[1]

*Corresponding author
Phone: 82-62-530-2142; Fax: 82-62-530-2149
E-mail: corhee@chonnam.chonnam.ac.kr

Abbreviation: LOX, lipoxygenase.

In spite of many reports of LOX components in higher plants, the enzymatic properties of these components, except those of soybean, were not studied much because most LOXs are not stable during purification.

We carried out some experiments on the LOX activitiies of black rice by the spectrophotometric method.[6] But we met some difficulties with opaque color of enzyme extracts. In this paper, we report the pH optimum and the effects of reaction temperature, substrate concentration, and storage temperature on LOX of black rice cultivars using oxygen monitoring system.

Materials and Methods

Materials. Three black rice cultivars(Chindo, Suwon-415, Yongkeum-1) harvested in 1997 were used in this experiment. Linoleic acid and ammonium sulfate were purchased from Sigma Chemical Company(St. Louis, MO, U.S.A.).

Preparation of enzyme extracts. Black rices were freshly powdered, and 10 g of this powder was stirred with 100 ml of 0.05 M phosphate buffer(pH 7.0) for 30 min at 4°C. The slurry was filtered through three layers of cheese-cloth and was centrifuged at 10,000 × g(Vision, Model VS-30000MT, Korea) for 30 min at 4°C. The supernatant was saturated with 30%(w/v) ammonium sulfate with continuous stirring for 30 min at 4°C. It was then centrifuged at 10,000 × g for 40 min at 4°C, and the precipitate was collected and dissolved in 1 ml of 0.05 M phosphate buffer.[7] This solution was used as the crude enzyme source.

Preparation of substrate. The linoleic acid stock solution used as the LOX substrate was prepared by the method of Axelrod.[1] This solution was prepared by pipetting 70 mg linoleic acid and Tween 20 into 25 ml of 0.2 M Tris-HCl

buffer(pH 9.0). The solution was homogenized with sonicator(Branson, Model B-221, Branson Cleaning Equipment Co., Sheiton, U.S.A.). One milliliter of stock solution was taken in a small vial, flushed with N_2 before closing, and stored at -40°C. Stock solution was diluted (1 : 5, v/v) with a buffer solution before use.

Determination of enzyme activity. Linoleic acid stock solution was diluted with 0.2 M Tris-HCl buffer(pH 9.0). The resulting concentration of linoleic acid was 2 mM. LOX activity was determined using an oxygen monitoring system (YSI, Model 5300, U.S.A.) to measure oxygen uptake. The reaction vessel was thermostated at 25°C with circulator (Jeiotech, Model VTRC-620, Korea). Two milliliters of the substrate solution was placed in the reaction vessel. After equilibrating the solution at 25°C for 5 min, the crude enzyme was added, and the oxygen uptake was measured. One unit of the activity was defined as 1 μmol of oxygen consumed per minute at 25°C. The velocity of reaction was determined using the linear region of the oxygen uptake curve.[5]

Optimum pH for enzyme activity. To determine the optimum pH, the activities of black rice's LOX were studied in the range of pH 6.0 to 9.0. The buffer systems[7] were 0.2 M phosphate buffer, pH 6.0 to 7.5 and 0.2 M Tris-HCl buffer, pH 8.0 to 9.0.

Protein analysis. Protein in the enzyme extracts was measured by the method of Lowry *et al.*[8] using bovine serum albumin as the standard protein.

Results and Discussion

Assay condition. Crude enzyme extracts had a optically opaque color caused by pigments of black rice. Therefore spectrophotometric method is not able to be applicable to this colored sample. Reaction velocity by oxygen polarographic method versus concentration of crude enzyme is plotted in Fig. 1. In the range of 300 mg/100 μl, the velocity had a linear relationship with enzyme concentration indicating the reaction was progressing with good reproducibility.

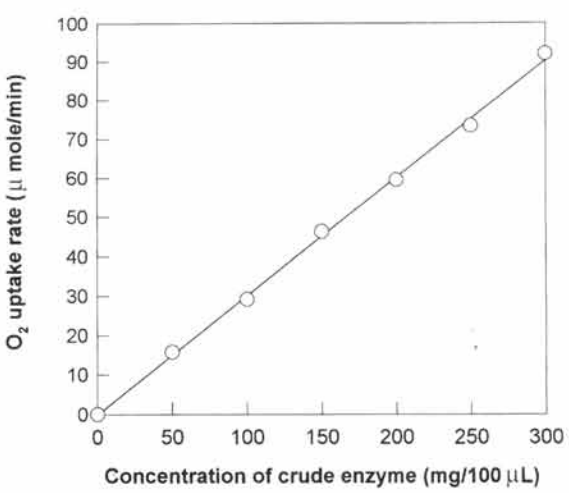

Fig. 1. Oxygen uptake rate vs amount of crude enzyme (Chindo). Assay ; T = 25°C, S = 2 mM linoleic acid, 0.2 M phosphate buffer(pH 7.0).

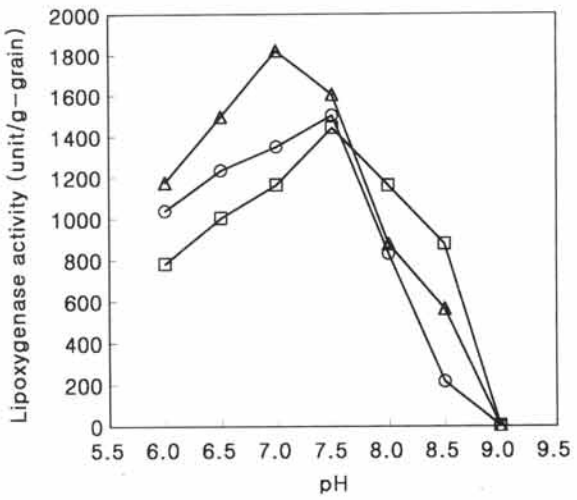

Fig. 2. pH activity profile for lipoxygenase from black rices. ○ : Chindo, △ : Suwon-415, □ : Yongkeum-1.

Table 1. Specific activity of lipoxygenase in three black rice cultivars

	Chindo	Suwon-415	Yongkeum-1
Total activity (unit/g-grain)	1502	1820	1440
Specific activity (unit/mg-protein)	41.0	27.3	29.6

Optimum pH. LOX activity was studied at different pH levels. As shown in Fig. 2, the optimum pH was about 7.0 for Suwon-415 and 7.5 for Chindo and Yongkeum-1. The pH optimum of 8.5 for rice bran LOX and 6.0 for soya bean meal LOX have been reported.[2,9] In most of the experiments for soya bean LOX, a bell-shaped curve with maximal activities between pH 7.0 and 8.0 have been found.[10] The specific activities of different cultivars determined at optimal pH is shown in Table 1. The activity of Yongkeum-1 LOX was observed with 1440 unit/g-grain. Since LOX acts on unsaturated fatty acids like linoleic acid, it can be assumed that Yongkeum-1 with lower activity of this enzyme has better storage qualities.[4] In case of specific activity, Suwon-415 had the lowest value with 27.3 unit/mg-protein where Chindo and Yongkeum-1 were 41.0 and 29.6 unit/mg-protein, respectively.

Effect of reaction temperature. The effect of reaction temperature for LOX activity was determined in 0.2 M phosphate buffer(pH 7.0) at 15~40°C. The results are presented in Fig. 3. In all the cultivars, there was an increase in the activity with increase in temperature. In case of Suwon-415, specially, the activity of LOX was rapidly increased with reaction temperature. The enzyme activities of Chindo and Suwon-415 at 40°C were 2.3 and 2.4 times

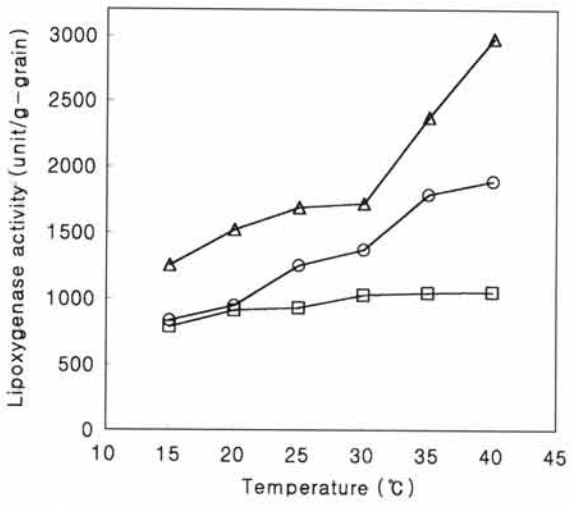

Fig. 3. Temperature activity profile for lipoxygenase from black rices. ○ : Chindo, △ : Suwon-415, □ : Yongkeum-1.

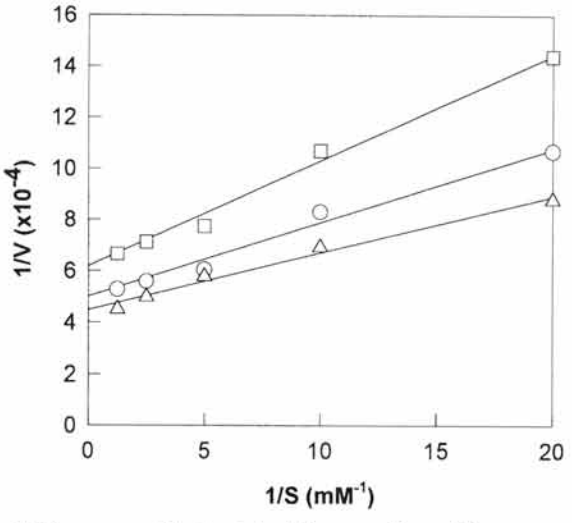

Fig. 4. Lineweaver-Burk plot of the reaction of lipoxygenase. ○ : Chindo, △ : Suwon-415, □ : Yongkeum-1.

Table 2. K_m and V_{max} values of lipoxygenase in three black rice cultivars.

	Chindo	Suwon-415	Yongkeum-1
K_m (mM)	0.059	0.050	0.066
V_{max} (unit/g-grain)	2020	2283	1616

higher than those at 15°C, respectively. But the enzyme activity of Yongkeum-1 at 40°C was 1.4 times higher than at 15°C. The optimum temperature for three major LOX isozymes of wheat germ was reported at about 45°C.[11] However, this was higher than those for soybeans, broad beans, and cowpeas with maximum activity at about 30°C.[12-14]

Effect of substrate concentration. Enzyme activity was tested at different concentrations(0.05~0.8 mM) of the substrate. The apparent K_m and V_{max} were determined from the Lineweaver-Burk plot(Fig. 4), and the results are

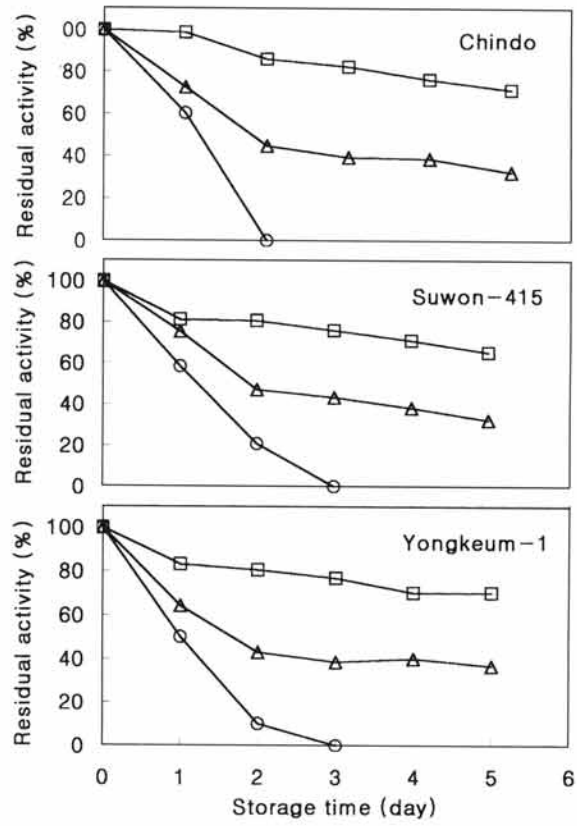

Fig. 5. Effect of storage temperatures on lipoxygenase activities of black rices. ○ : 25°C, △ : 4°C, □ : -40°C.

summarized in Table 2. The extrapolated apparent K_m values of Chindo, Suwon-415, and Yongkeum-1 were 0.059, 0.050, and 0.066 mM with linoleic acid as the substrate, respectively. The corresponding values for the enzymes of soya bean,[15] rice bran[2], and wheat[16] are 1.00, 0.35, and 5.00 mM, respectively. For the potato enzyme, K_m values of 0.3 and 0.8 mM have been reported[16]. The V_{max} values of Chindo, Suwon-415, and Yongkeum-1 were 2020, 2283, and 1616 unit/g-grain, respectively. Kim *et al.*[17] reported that the V_{max} values of *Tongjine, Kumoh, and Kanchukbyeo* rice cultivars were 57.89, 19.85, and 31.38 unit/mg-protein, respectively.

Stability of the enzyme extracts. Crude enzyme extracts were stored at 25, 4, and -40°C and the activities were tested every 4 hr for 5 days. The results are presented in Fig. 5. In all the cultivars, most of the LOX activities disappeared after 2 days storage at 25°C. There is not so much difference between three cultivars in the stability of enzyme extracts. In the case of storage at 4°C, LOX activity, however, dropped during the first 2 days storage and then stabilized at 40% until 5 days. Residual activity was about 70% when crude enzyme extracts were stored at -40°C after 5 days. Shastry *et al.*[2] reported that LOX of unfractionated rice bran was optimally active at pH 8.5 and was stable for 15 days at 3~5°C.

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