Purification and Characterization of Lipoxygenase Isoenzymes in Germinating Barley[1]

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ABSTRACT

Two lipoxygenase (LOX) isoenzymes were extracted from barley (*Hordeum vulgare* 'Robust') and purified by hydroxylapatite chromatography, yielding LOX-1 with a pI of 5.2 and LOX-2 with a pI of 6.7. LOX-1 and LOX-2 appear to be monomeric proteins of 90 and 95 kDa, respectively. LOX-1 converted linoleic and linolenic acids into mainly 9-hydroperoxides, whereas LOX-2 produced mainly 13-hydroperoxides. Linoleic acid was found to be the best substrate for both isoenzymes. K_m values for this substrate were determined to be 1.3×10^{-5} *M* for LOX-1 and 1.9×10^{-5} *M* for LOX-2. LOX-2 can oxidize esterified derivatives of linoleic acid (methyl linoleate and trilinolein) more readily than LOX-1 can. LOX-2 also had more heat resistance and better stability than LOX-1 did. LOX-2 developed only after germination, and the activity of both LOX-1 and LOX-2 increased to a large extent during germination. LOX-1 was localized exclusively in the germ in sound barley. Upon germination, LOX-1 and LOX-2 developed in newly synthesized rootlet and acrospire tissue. The rootlets contained exclusively LOX-2, while the acrospire contained both isoenzymes.

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Lipoxygenases (linoleate:oxygen oxidoreductase EC 1.13.11.12) are a group of enzymes that catalyze the dioxygenation of polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene system to produce conjugated *cis, trans*-diene hydroperoxy derivatives. Lipoxygenases (LOX) are particularly relevant to plants used for foods and beverages because their action converts the essential polyunsaturated fatty acids into hydroperoxides, which can decompose to form compounds with characteristic aromas and flavors. *Trans*-2-nonenal, which is formed from the 9-hydroperoxide of linoleic acid, has been shown to be, in part, responsible for the development of a stale flavor in beer during storage (Jamieson and Van Gheluwe 1970, Dominquez and Canales 1974). Drost et al (1990) demonstrated a strong correlation between the LOX activity in malt and the nonenal potential in corresponding worts. They suggested that LOX may have a determining role in the staling process.

Because LOX levels are cultivar-related (Schwarz and Pyler 1984), selection of cultivars inherently low in LOX levels may be one means of controlling beer staling. LOX activity might also be limited during malting and mashing by altering process parameters or equipment, such as kiln temperature, mashing temperature and pH, oxygen pick-up during mashing, and wort separation (Drost et al 1990). Knowledge of the fundamental characteristics of barley LOX isoenzymes and their activity during malting and mashing may aid the maltster and brewer in developing means to limit such activity.

LOX in barley was first reported by Frank and Freshse (1953). Thereafter, many attempts were made to characterize the enzyme (Graveland et al 1972; Furling 1975; Yabuuchi and Amaha 1975; Yabuuchi 1976; Lulai and Baker 1975, 1976; Heiman and Timm 1977; Lulai et al 1981; Baxter 1982; Van Aarle et al 1991; Doderer et al 1992). However, most of that research dealt only with LOX-1, which exists in sound barley. Yabuuchi (1976), Baxter (1982), and Doderer et al (1992) have reported the existence of a second isoenzyme (LOX-2), which generally appears to develop only after germination.

The objective of this research was to purify and characterize the barley LOX isoenzymes. A portion of this research is contrasted with results recently reported by Doderer et al (1992). Factors involving temperature, storage stability, and tissue distribution of the LOX isoenzymes have not been previously reported. A simple method for separation and purification of the LOX isoenzymes is also described.

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MATERIALS AND METHODS

Plant Material

The six-rowed malting barley (*Hordeum vulgare*) cultivar, Robust, was used throughout this study because it is currently one of the most widely grown malting cultivars in the United States. The sample from the 1990 crop year was obtained from the North Dakota State University Agricultural Experiment Station, Fargo, ND. The sample contained 13.8% protein and 8.3% moisture and appeared to be free of weathering.

Chemicals

Sephacryl S-300 and diethylaminoethyl Sepharose CL-6B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Protein molecular weight standards (for both gel filtration and electrophoresis), pI marker proteins, linoleic acid, linolenic acid, arachidonic acid, methyl linoleate, trilinolein, Triton X-100 (reduced), blue dextran, soybean lipoxygenase (type IV), and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide were obtained from Sigma Chemical Company (St. Louis, MO). Hydroxylapatite gel (Bio-Gel HT), ampholytes (pH 4–6, pH 5–8, and pH 3–7), Coomassie Blue stain, acrylamide, bisacrylamide, and sodium dodecyl sulfate were obtained from Bio-Rad (Richmond, CA). Platinum oxide was obtained from Aldrich Chemical Company (Milwaukee, WI).

Germination

Barley samples (60 or 80 g) were steeped to 44% moisture at 16°C. Two 2-hr air rests were included for each 24 hr of steep time. Germination was conducted in a 400-ml beaker at 16°C and 85% rh. Samples were removed from the germination cabinet daily, and the entangled rootlets were separated by hand. At this time, distilled water was added to the germinating sample to replace any moisture loss.

Substrate Preparation

Linoleic acid and linolenic acid substrates were prepared according to the method of Surrey (1964). Substrate was frozen under an atmosphere of nitrogen until needed. The final concentration of linoleic acid was approximately 8 m*M*.

Enzyme Extraction

LOX extracts were prepared daily, before assay, according to the method of Lulai and Baker (1976). Ground samples were extracted for 1 hr at 4°C with 0.1*M* potassium phosphate buffer, pH 6.0.

Enzyme Assay

LOX activity was determined by measuring the increase in absorbance at 234 nm, according to a modification of the method of Vick and Zimmerman (1976). In a typical assay, 0.980 ml of 0.1*M* potassium phosphate buffer (pH 6.0) and 0.01 ml of freshly prepared enzyme extract were mixed in a 1-ml cuvette.

The reaction was initiated by the addition of 0.01 ml of recently thawed substrate (8 m*M*). For the assay of fractions collected from different columns, larger volumes of enzyme extract (0.01–0.1 ml) were used. One unit of LOX activity was defined as 1 μmol of hydroperoxide formed per minute at 25°C. Hydroperoxide-metabolizing enzymes in chromatographic fractions were detected by monitoring the decrease in absorbance at 234 nm, when linoleic acid hydroperoxides were used as the substrate (Zimmerman and Vick 1970).

Protein Assay

For the chromatography fractions, the relative protein content was determined by measuring the absorbance at 280 nm. For calculation of enzyme purification, protein was assayed by the Coomassie blue dye binding method (Bio-Rad). Bovine plasma gamma globulin (200–1,400 μg/ml) was used as a standard. Absorbance was measured at 595 nm.

Enzyme Purification

Seventy milliliters of crude enzyme extract from four-day germinated barley was fractionated by addition of solid ammonium sulfate. The precipitate at 20% saturation, which contained no significant LOX activity, was discarded. The supernatant, containing most of the LOX activity, was precipitated by addition of ammonium sulfate to 50% saturation. The pellet obtained by centrifugation was resuspended in 10 ml of 10 m*M* potassium phosphate, pH 6.0. Approximately 60% of the LOX activity was recovered by ammonium sulfate precipitation.

The fraction obtained from ammonium sulfate precipitation was applied to a diethylaminoethyl Sepharose CL-6B ion-exchange column (2.6 × 20 cm) equilibrated with 50 m*M* potassium phosphate, pH 6.5. Proteins were eluted by a linear gradient of sodium chloride (0–0.2*M*) in the same buffer at a flow rate of 1.6 ml/min. All operations were conducted at 4°C. Fractions of 3 ml were collected. Fractions with active LOX were combined, and ammonium sulfate was added to 55% saturation to precipitate LOX. After centrifugation (12,000 × *g*, 10 min) the pellet was resuspended in 2 ml of 10 m*M* potassium phosphate buffer, pH 6.5.

The partially purified sample was applied to a Sephacryl S-300 gel-filtration column (1.5 × 100 cm) equilibrated with 100 m*M* potassium phosphate, pH 6.5. The enzyme was eluted with the same buffer at a flow rate of 0.63 ml/min. Eighty fractions of 2.8 ml were collected. In order to estimate the molecular weights of each isoenzyme, five protein standards with known molecular weights were applied to the same column. The protein markers were apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The fractions with LOX activity were combined, precipitated (55% saturation with ammonium sulfate), and resuspended in 2 ml of 10 m*M* potassium phosphate, pH 6.5.

The enzyme fraction purified by gel filtration was applied to a hydroxylapatite column (1.0 × 40 cm). The column was equilibrated with 10 m*M* potassium phosphate, pH 6.5, containing 0.1% Triton X-100. Detergent was used to improve separation of LOX-1 and LOX-2. A linear gradient of potassium phosphate (10–500 m*M*) was used for elution. The flow rate was controlled at 0.7 ml/min, and 5.0-ml fractions were collected. LOX-1 and LOX-2 were completely resolved. Fractions of LOX-1 and LOX-2 were concentrated with Centricon 30 microconcentrators (Amicon, Beverly, MA) to the desired volume. The purified LOX isoenzymes were used for studies involving gel electrophoresis.

For all other studies, LOX isoenzymes were purified and separated directly on the hydroxylapatite column after precipitation with ammonium sulfate (to 50% saturation) as previously described.

Enzyme Distribution

Barley that had been germinated for five days was hand-dissected to remove the acrospire, rootlets, and germ. Each tissue, as well as the remaining caryopsis, was extracted with 100 m*M* potassium phosphate, pH 6.0. Total LOX activity in the crude extract of each tissue was determined. The distribution of the LOX isoenzymes was calculated after separation on the hydroxylapatite column.

Substrate-Product Specificity

One milliliter of linoleic or linolenic acid (8 m*M*) was incubated with 1.0 ml of partially purified LOX-1 and LOX-2 obtained by ammonium sulfate precipitation and hydroxylapatite chromatography. Samples were incubated for 10 min, and reactions were stopped by adjusting the pH to 3.0–3.5 with 1*N* HCl. The hydroperoxides formed were extracted with a Prep Sep C_{18} solid-phase extraction column (Fisher Scientific, Fair Lawn, NJ) and purified by thin-layer chromatography on silica gel G with fluorescent indicator. The solvent system used was a mixture of hexane, diethyl ether, and acetic acid (60:40:1, v/v). Hydroperoxides on the thin-layer chromatography plate were identified under ultraviolet light. The purified hydroperoxides were then methylated with diazomethane, reduced with sodium borohydride, hydrogenated, and silylated by reacting with bis(trimethylsilyl)trifluoroacetamide according to the method of Vick (1975). A Hewlett-Packard HP-5890 gas chromatograph and HP-5970 series mass-selective detector were used for gas chromatography-mass spectrometry analysis. An HP-1 cross-linked methyl silicone gum column (25-m × 0.2-mm × 0.33-μm film thickness) was used. The temperature was programmed from 50 to 280°C at 10°C/min. The products (13- or 9-hydroperoxides) were identified by their mass spectra. The percent of each hydroperoxide was determined by selected ion monitoring of the fragments resulting from cleavage on either side of the trimethylsilyl group (Vick and Zimmerman 1981). These fragments had mass-to-charge ratio (*m/z*) 173 and 315 for the 13-hydroperoxide, and *m/z* 229 and 259 for the 9-hydroperoxide. The sum of the peak areas produced by *m/z* 173 and 315 were compared to the sum of peak areas of *m/z* 229 and 259 to determine the percent of each isomer.

Electrophoresis

The molecular weights of the LOX isoenzymes were also estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking gel was 5% polyacrylamide in tris-glycine buffer (pH 6.6) containing 1% SDS, and the running gel was 7% polyacrylamide gel in tris-glycine buffer (pH 8.8) containing the same percentage of SDS. Protein samples (500–1,000 μg/20 μl) were treated with SDS before electrophoresis. Electrophoresis was conducted for 60 min at 100 V for the stacking gel and at 200 V for the running gel. Gels were stained for 30 min with Coomassie brilliant blue R-250 (0.25%, w/v) in a solvent containing 45% methanol and 10% acetic acid (v/v); they were destained in a solvent composed of 5% methanol and 7% acetic acid (v/v) for several hours. The molecular mass markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The molecular weights of LOX-1 and LOX-2 were approximated by comparing the mobility of LOX-1 and LOX-2 with the molecular markers.

Isoelectric Focusing

Isoelectric focusing of LOX extracts obtained by ammonium sulfate precipitation was performed using a Rotofor preparative isoelectric focusing apparatus (Bio-Rad) according to the instructions of the manufacturer. Ampholytes used were: Bio-lyte 4/6, 0.8%; Bio-lyte 5/8, 0.8%; and Bio-lyte 3/10, 0.8%. NaOH (0.1*M*) and H_3PO_4 (0.1*M*) were used as anode and cathode electrode solutions. Focusing was conducted at 4°C for 5 hr at 12 W constant. The focused samples were then collected in 20 fractions of 2.5 ml. The LOX activity of each fraction was measured spectrophotometrically using linoleic acid (80 μ*M*) as the substrate.

RESULTS AND DISCUSSION

Isoenzyme Purification

A summary of the purification steps of LOX from four-day germinated barley is shown in Table I. LOX-2 was purified approximately 260-fold with a specific activity of 76 units per milligram of protein. LOX-1 was purified 120-fold with a specific activity of 36 units per milligram of protein. Approximately equal amounts (0.9 mg) of each isoenzyme were obtained. Hydroperoxide-metabolizing enzymes (hydroperoxide dehydrase or hydroperoxide lyase) were eliminated by gel filtration (M_r in excess of 200,000 kDa)(Fig. 1). LOX-1 and LOX-2 were separated by hydroxylapatite chromatography in the final step. LOX-1 eluted at 0.05 *M* potassium phosphate, and LOX-2 eluted at 0.18 *M* (Fig. 2). The highly purified LOX preparations were used for studies involving gel electrophoresis and isoelectic focusing.

For all other studies, the LOX isoenzymes were separated and purified by ammonium sulfate precipitation, followed by chromatography on hydroxylapatite. LOX-1 was purified 25-fold with a 30% recovery by this two-step method. LOX-2 was purified 30-fold with 40% recovery. LOX isoenzymes eluted at the buffer concentrations listed above. Hydroperoxide-metabolizing enzymes eluted at a much higher buffer concentration.

Substrate-Product Specificity

Both LOX-1 and LOX-2 were compared spectrophotometrically for relative specificity with regard to five different substrates: linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidonic acid ($C_{20:4}$), methyl linoleate, and trilinolein, each at a concentration of 80 μM. Potassium phosphate buffer, 0.1 *M* (pH 6.5) was used for the assay. Results are presented in Figure 3. Linoleic acid proved to be the preferred substrate for both isoenzymes. For LOX-1, activity with linolenic acid was about 90% of that for linoleic acid. In contrast, LOX-2 converted linolenic acid at relatively slower rate (72%) than that of linoleic acid. The results also indicated that LOX-2 could catalyze the oxidation of esterified derivatives of linoleic acid such as methyl linoleate and trilinolein at a much faster rate than LOX-1 could.

Confirmation of hydroperoxide isomers formed after reaction with linoleic and linolenic acids was accomplished by gas chromatography-mass spectrometry. The mass spectra of trimethylsilylated methyl hydroxystearate derivatives of 9- and 13-hydroperoxides are shown in Figure 4. Clear diagnostic ion fragments at m/z 259 and m/z 229 were characteristic of methyl 9-OTMS stearate; fragments at m/z 173 and m/z 315 were characteristic of methyl-13-OTMS stearate. Selected ion monitoring of m/z 173, 229, 259, and 315 (Fig. 5) showed that LOX-1 primarily catalyzed the formation of the 9-hydroperoxide (the

TABLE I
Purification Steps of Lipoxygenase Isoenzymes LOX-1 and LOX-2 from Four-Day Germinated Barley

Step	Protein (mg)	Activity (units)[a]	Specific Activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	903	267	0.295	100	1.0
$(NH_4)_2SO_4$, 20–50%	220	176	0.788	65.8	2.7
Diethylaminoethyl CL-6B	58	164	2.83	61.6	9.6
Sephacryl S-300	10	145	14.5	54.2	49.0
Hydroxylapatite					
LOX-1	0.85	30.5	35.9	11.4	122.0
LOX-2	0.92	69.5	76.0	26.1	258.0

[a] One unit of activity is defined as 1 μmol of hydroperoxide formed per minute at 25° C.

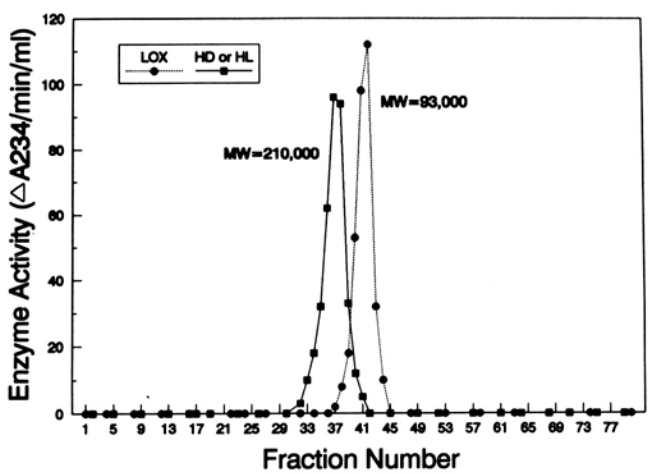

Fig. 1. Separation of lipoxygenase (LOX) isoenzymes from hydroperoxide metabolizing (hydroperoxide dehydrase [HD] or hydroperoxide lyase [HL]) enzymes on a Sephacryl S-300 chromatography column (1.6 × 100 cm). Fractions of 2.8 ml were collected.

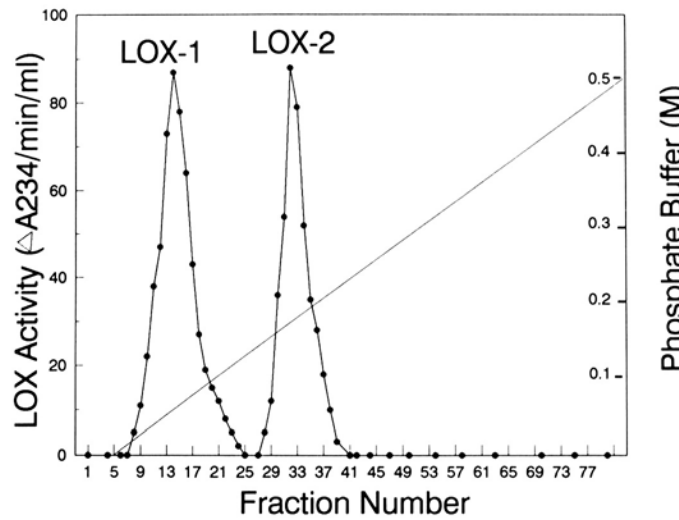

Fig. 2. Separation of two lipoxygenase isoenzymes, LOX-1 and LOX-2, from barley germinated for five days on a hydroxylapatite chromatography column (1 × 40 cm). Isoenzymes were eluted with a gradient of potassium phosphate (10–500 m*M*). Fractions of 5.0 ml were collected.

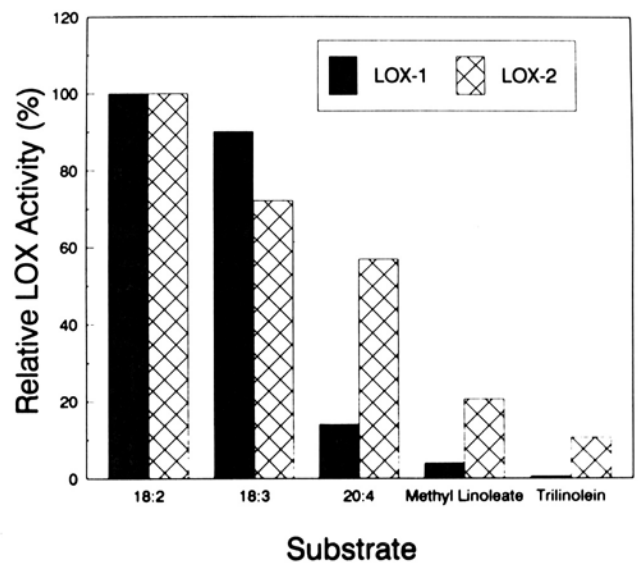

Fig. 3. Relative substrate specificities of lipoxygenase isoenzymes, LOX-1 and LOX-2. The same concentration (80 μM) was used for all the substrates. Substrates: linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidonic acid ($C_{20:4}$). LOX activity with linoleic acid was designated as 100%.

ratio 9- to 13-hydroperoxide was 80:20 for both $C_{18:2}$ and $C_{18:3}$). LOX-2 yielded primarily 13-hydroperoxide (the ratio 9- to 13-hydroperoxide was 37:63 for $C_{18:2}$ and 47:53 for $C_{18:3}$). Thus, for LOX-1, the ratio 9- to 13-isomers was not affected by substrate

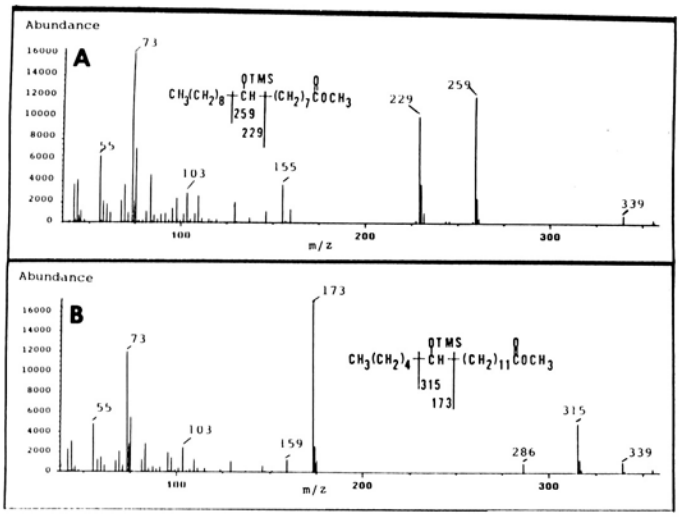

Fig. 4. Partial mass spectra of (**A**) methyl 9-OTMS and (**B**) methyl 13-OTMS stearate derivatives resulting from hydrogenation-reduction (H_2-PtO_2) of hydroperoxides of linoleic acid generated by incubation with partially purified lipoxygenase isoenzymes, LOX-1 and LOX-2, respectively.

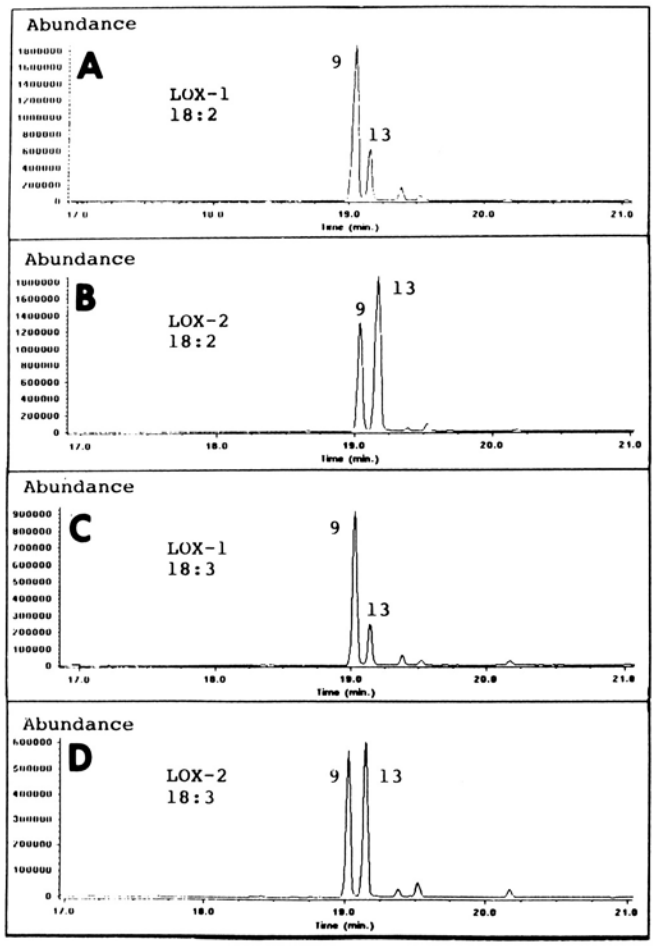

Fig. 5. Gas chromatography-mass spectrometry chromatograms of the hydroperoxides of linoleic ($C_{18:2}$) and linolenic acids ($C_{18:3}$) after incubation of $C_{18:2}$ and $C_{18:3}$ with lipoxygenase isoenzymes, LOX-1 or LOX-2. **A**, LOX-1 with $C_{18:2}$. **B**, LOX-2 with $C_{18:2}$. **C**, LOX-1 with $C_{18:3}$. **D**, LOX-2 with $C_{18:3}$.

($C_{18:2}$ or $C_{18:3}$), but LOX-2 produced more 13-hydroperoxide when linoleic acid was used as the substrate.

Using a gravimetric procedure, Graveland et al (1972) incubated a quiescent barley flour fraction with a solution of linoleic acid to produce a 9- to 13-hydroperoxylinoleic acid ratio of 8:1. Yabuuchi and Amaha (1975) reported a similar ratio (9:1) by analyzing the ratio of the diagnostic ion for each isomer after gas chromatography-mass spectrometry of nonsilylated hydroxystearate methyl esters. Using the same method, Yabuuchi (1976) also reported that ratios of 9- to 13-hydroperoxides varied from 96:4 for ungerminated barley to 70:30 for barley germinated for seven days. This method was questioned by Lulai (1978) because sodium borohydride reduction followed by hydrogenation produces chemical isomerization, and nonsilylated hydroxystearates do not give as reliable quantitative results by mass spectrometry as do trimethysilyl esters.

Lulai (1978) studied LOX specificity in quiescent and germinated barley by gas chromatography-mass spectrometry of trimethylsilylated hydroxystearates. He reported that the ratio 9- to 13-hydroperoxide changed from 2.3:1 for quiescent barley to 0.7:1 for barley germinated for four days. However, none of the previous work showed partially purified LOX-1 and LOX-2 isoenzymes used as an enzyme source. The enzyme used in previous studies was actually a mixture of LOX-1 and LOX-2.

Molecular Mass Determination

Gel filtration resolved LOX-1 and LOX-2 as a single peak with an approximate molecular mass of 93 kDa (Fig. 1). SDS PAGE, however, appeared to resolve LOX-1 and LOX-2 as two separate bands with molecular masses of 90 and 95 kDa, respectively (Fig. 6). The identity of LOX isoenzymes in the SDS-PAGE gel was tentatively confirmed by performing SDS-PAGE individually on the highly purified isoenzymes.

pH Optimum

The activity of LOX-1 and LOX-2, as affected by pH, was determined with three buffers of the same molarity (0.1*M*), using linoleic acid as the substrate. The pH optima are shown in Figure 7. LOX-1 exhibited a pH optimum at 6.3, whereas LOX-2 had optimum activity at pH 6.0 and a secondary pH optimum at 8.0. Vick and Zimmerman (1976) also reported two pH optima for LOX from watermelon seedlings. Yabuuchi (1976) indicated that barley LOX-2 had a wider pH optimum (7.0–7.5); LOX-1 exhibited optimal activity at pH 7.5. Other reports on LOX-1 from sound barley indicated a pH optimum at 5.9–6.0 (Lulai

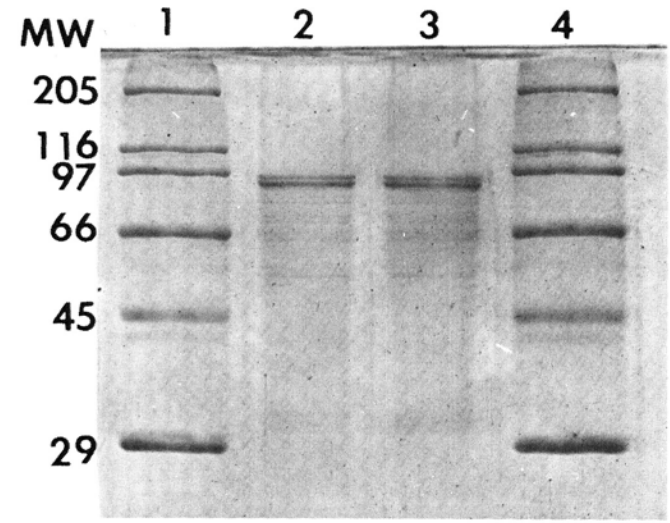

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lipoxygenase isoenzymes (LOX) obtained from germinated barley. Lanes 1 and 4, molecular weight markers. Lanes 2 and 3, partially purified LOX obtained from germinating barley. Major bands in lanes 2 and 3 tentatively identified as LOX-2 (95 kDa) and LOX-1 (90 kDa).

and Baker 1975) and 6.5 (Fuhrling 1975). Doderer et al (1992) demonstrated the same pH optimum for both LOX-1 and LOX-2, but they indicated that LOX-1 had a broader optimum pH range than LOX-2.

Isoelectric Point

The pI values of LOX-1 and LOX-2 were determined as 5.2 and 6.7, respectively, with the Rotofor isoelectric focusing apparatus (Fig. 8). Yabuuchi (1976) determined the pI values of 4.9 and 6.6 for LOX-1 and LOX-2, respectively, using preparative isoelectric focusing apparatus. Doderer and coworkers (1992) reported pI values of 5.2 and 6.8 for LOX-1 and LOX-2, respectively.

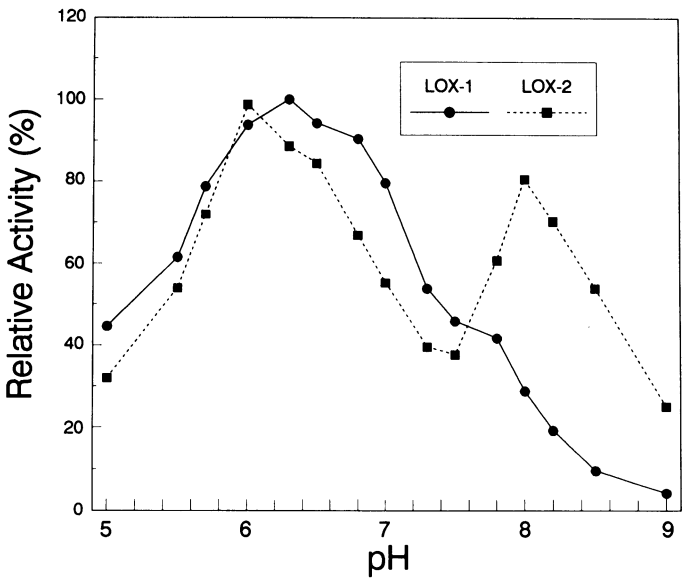

Fig. 7. pH optima of lipoxygenase isoenzymes LOX-1 and LOX-2. The reactions were conducted with 80 μM linoleic acid as substrate in buffers (acetate pH 5.0–5.8, phosphate pH 6.0–7.8, Tris pH 8.0–9.0) of constant molarity (0.1 M).

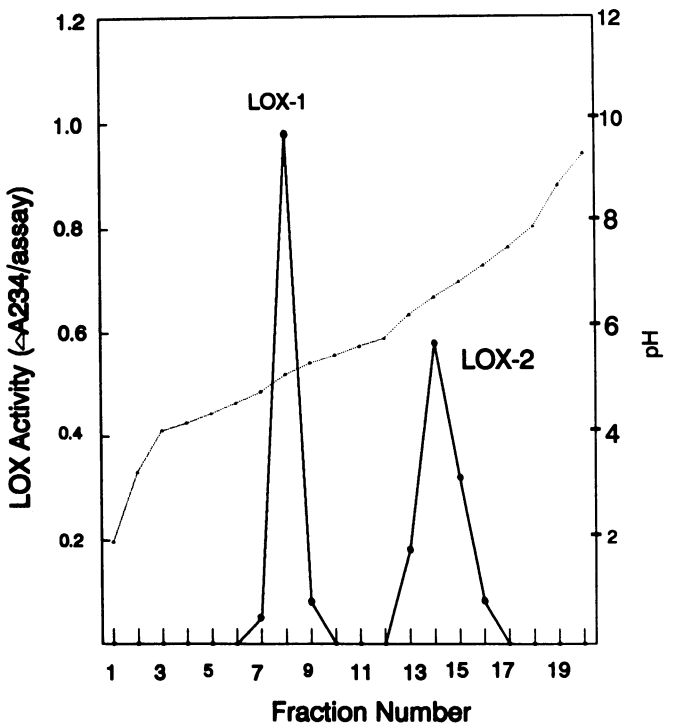

Fig. 8. Separation of lipoxygenase isoenzymes (LOX) from germinating barley on the Rotofor isoelectric focusing apparatus. The isoelectric points of LOX-1 and LOX-2 were determined as 5.2 and 6.7, respectively.

Kinetic Parameters

The apparent K_m and V_{max} were determined using seven concentrations of linoleic and linolenic acids (6–80 μM). Lineweaver-Burk reciprocal plots are shown in Figure 9. The results indicated that linoleic acid was preferred over linolenic acid as a substrate for LOX-1 and LOX-2, and that this preference was more pronounced for LOX-2. In general, LOX-1 had relatively lower K_m and V_{max} values than LOX-2. The apparent K_m values for linoleic acid of LOX-1 and LOX-2 were 1.3 and 1.9×10^{-5} M. This result is close to the value of $1.5–1.8 \times 10^{-5}$ M reported by Doderer et al (1992). The turnover numbers of LOX-1 and LOX-2 for linoleic acid were calculated as 3.3 and 4.8×10^5, respectively.

Effect of Temperature

A temperature denaturation curve was plotted for LOX-1 and LOX-2 (Fig. 10). Samples were heated for 5 min at the indicated temperatures. The results indicated that LOX-2, which developed during germination, was more heat resistant than was LOX-1. Poca et al (1990) reported similar results for LOX isoenzymes in maize. The heat resistance of LOX-2 might offer better opportunity for it to survive the final stages of malting, and, thus, it may play a significant role in mashing. However, the activity of the two isoenzymes during malting and mashing should be determined. Baxter (1982) found that the small amount of total LOX activity that survived kilning was more stable towards further heating, and almost 60% of the activity survived 1 hr at 67°C.

Storage Stability

The results of stability experiments (Fig. 11) showed that LOX-2 is much more stable than LOX-1 when these isoenzymes are stored at 25°C or at 4°C. LOX-2 retained 80% of its original activity after storage at 4°C for one week, whereas LOX-1 retained only about 50% of its activity. Large differences in stability between the two isoenzymes were also found in storage at room tempera-

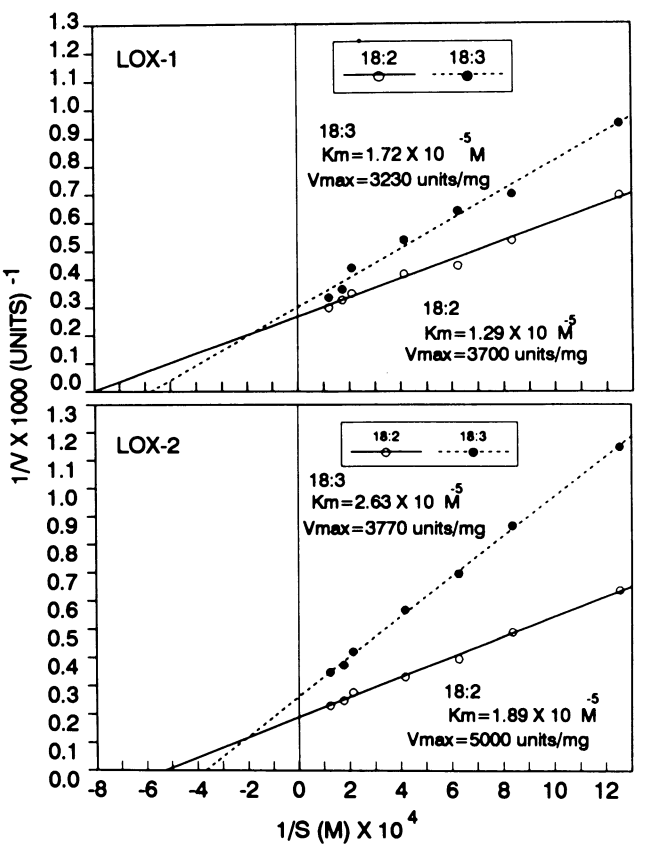

Fig. 9. Lineweaver-Burk plots and kinetic parameters of lipoxygenase isoenzymes (LOX-1 and LOX-2) using linoleic ($C_{18:2}$) and linolenic acids ($C_{18:3}$) as substrates.

ture. LOX-1 lost more than 60% of its activity during the first day of storage at room temperature, while only 13% of LOX-2 activity was lost. Baxter (1982) reported similar results regarding the stability of LOX isoenzymes. Both isoenzymes were moderately stable when frozen and stored at −20°C. It was also noted that the stability of both isoenzymes was somewhat dependant on the enzyme concentration: the higher the concentration, the poorer the stability. Partially purified enzyme preparations were more stable than crude enzyme extracts. This could indicate the existence of proteases or inhibitory materials in the crude enzyme extracts.

Development of LOX Isoenzymes in Germinating Barley

Sound barley exhibited only LOX-1 when the 20–50% $(NH_4)_2SO_4$ saturation precipitate of the crude extract was applied to a hydroxylapatite column. LOX-2 was detected only in germinating barley. The activity of both LOX-1 and LOX-2 increased during germination (Table II). Approximately equal activities of LOX-1 and LOX-2 were detected in barley that was germinated for five days (Fig. 1). Previous studies indicated that LOX levels change during germination (Lulai et al 1981, Baxter 1982, Schwarz and Pyler 1984). However, no detailed work was conducted concerning changes in the individual LOX isoenzymes during germination. Yabuuchi (1976) reported that only LOX-1 was found in sound barley, and that secondary LOX-2 activity developed during germination (cv. Satsuki Nijo). His results also indicated that the activity of LOX-2 was much weaker than LOX-1 after four days of germination. However, he did not indicate whether the activity of LOX-1 increased during germination. Baxter (1982) reported that both LOX-1 and LOX-2 were present in sound barley, as well as in green malt (cv. Nudinka). The ratio of LOX-1 to LOX-2 was 20 in barley, falling to approximately 4 in barley that had been germinated for four days. She concluded that the activity of both isoenzymes increased during germination, but LOX-2 increased to a greater extent. The presence of LOX-2 in ungerminated barley may be a genetic factor. Van Aarle et al (1991) reported the presence of LOX-2 in the cultivar Blenheim but not in the cultivar Triumph. The genotypic variation of LOX isoenzymes is currently under investigation in our laboratory.

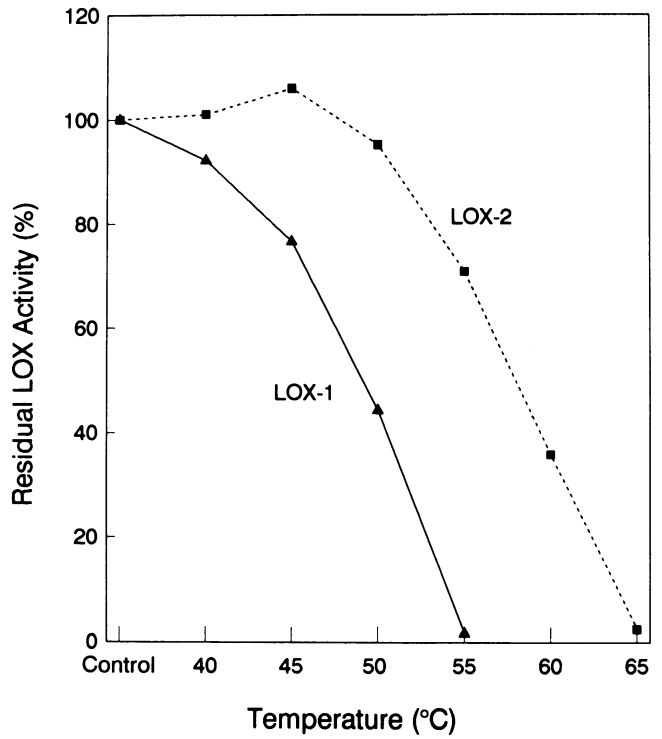

Fig. 10. Effect of 5-min exposures to various temperatures on the activity of lipoxygenase isoenzymes (LOX-1 and LOX-2).

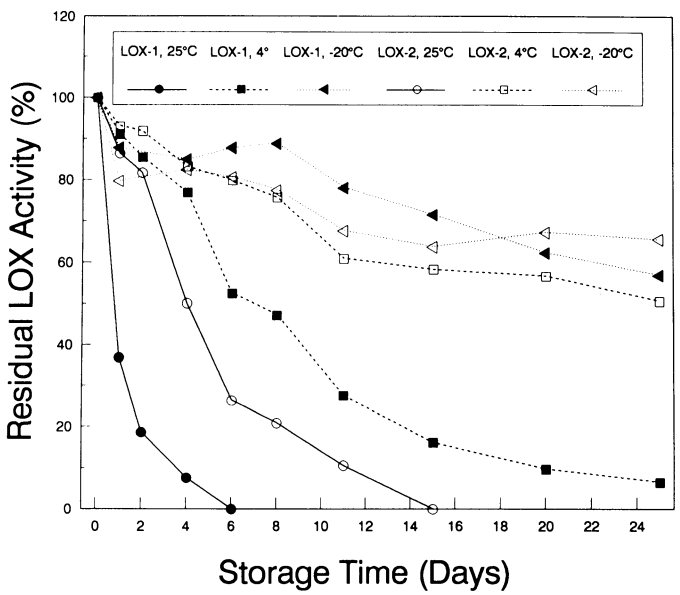

Fig. 11. Stability of lipoxygenase isoenzymes (LOX-1 and LOX-2) extracts stored at 25, 4, and −20°C.

LOX Distribution

The results shown in Table III indicated that the LOX activity was primarily located in the germ, especially in the newly synthesized acrospire and rootlet tissues. The fact that there was no LOX-2 in the germ may indicate that the LOX activity in germ was caused by the original LOX-1 in sound barley. The activity that increased during germination (LOX-1 and LOX-2) may have been primarily distributed in the newly synthesized tissues. The LOX activity in the endosperm-pericarp-husk could be partially caused by the incomplete removal of germ, acrospire, and rootlets.

SUMMARY AND CONCLUSIONS

Two LOX isoenzymes in germinating barley have been reported (Yabuuchi 1976, Baxter 1982, Doderer et al 1992). Methods reported previously for the separation of these two isoenzymes were preparative isoelectric focusing (Yabuuchi 1976) and S-Sepharose Fast Flow chromatography (Doderer et al 1992). Although preparative isoelectric focusing was an effective method

TABLE II
Lipoxygenase Isoenzyme (LOX-1 and LOX-2) Development During Germination

Germination Time (days)	Activity (μmol min^{-1} g^{-1} of fresh weight)[a]	
	LOX-1	LOX-2
0 (Sound barley)	4.3	nd[b]
1	7.5	2.9
3	9.9	7.5
5	13.1	11.8

[a]45% moisture basis.
[b]Not detectable.

TABLE III
Distribution of Lipoxygenase Isoenzymes LOX-1 and LOX-2 in Five-Day Germinated Barley

Tissue	Weight (g)	Total Activity (units)	Weight/Activity (units/g)	Ratio
Acrospire	5.71	307	53.8	58:42
Rootlets	5.01	240	47.9	0:100
Germ	8.30	200	37.7	100:0
E/P/H[a]	47.87	129	2.7	nd[b]

[a]Endosperm/pericap/husk.
[b]Not detectable.

for resolving LOX isoenzymes, the activity of each isoenzyme was much reduced. Four LOX peaks were resolved by S-Sepharose chromatography (Doderer et al 1992), and it was concluded that peak 1 and peak 2 were the same as LOX-1 and that peak 3 and peak 4 were the same as LOX-2. In the present study, a simple hydroxylapatite chromatography method was developed by which the two isoenzymes were completely separated, preserving much of the activity of each isoenzyme. The separation of LOX isoenzymes by hydroxylapatite was not reported previously. The present, as well as previous, research work demonstrated distinct differences between barley LOX-1 and LOX-2. These include pI values, storage and heat stabilities, substrate and product specificities, pH optima, and kinetic parameters. The two isoenzymes were shown to have similar molecular mass.

The LOX activity that developed during germination appeared to be largely localized in the newly synthesized acrospire and rootlets. Rootlet tissue, which accounted for 27.4% of the total LOX activity, contained LOX-2 exclusively. This is of interest as the rootlets are removed after the preparation of malt. LOX-2 catalyzes mainly the formation of 13-hydroperoxides. As LOX-2 appeared to be more heat-stable than was LOX-1, LOX-2 may have greater potential to survive the final stages of malting and even into mashing. However, LOX-1 should be of interest to maltsters and brewers as it catalyzes primarily the formation of the 9-hydroperoxide. The 9-hydroperoxide of linoleic acid is a probable precursor of *trans*-2-nonenal. Continued research to investigate the extent of survival of each isoenzyme during kilning and mashing is in progress.

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