Purification and Characterization of Lipoxygenase Isozymes from Wheat Germ

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ABSTRACT

To investigate the mechanism by which lipoxygenase improves the rheological properties of wheat flour doughs, wheat lipoxygenase was extracted first with acetate buffer from wheat germ and fractionated by ammonium sulfate. The lipoxygenase fraction was further purified by diethylaminoethyl (DEAE)-Sepharose chromatography and then separated into three major isozymes (L-1, L-2, and L-3) and a minor isozyme (L-a) by carboxymethyl-Sepharose chromatography. Each isozyme was purified by gel filtration and DEAE-Sepharose chromatography. Sodium dodecyl sulfate-polyacrylamide gel chromatography of these purified isozymes exhibited a single band with almost the same retardation factor. These purified lipoxygenase isozymes were characterized by enzymatic properties (pH activity profile, thermal sensitivity, substrate specificity, effect of metal ions), amino acid composition, and their effects on wheat flour. After reaction with each purified lipoxygenase isozyme, the treated flour exhibited higher foaming activity and lower contents of the sulfhydryl group than did the control flour, which received no enzyme treatment. The treated flour also showed a shorter dough development time and less resistance after peak time on a mixogram than did the control flour. The flour treated with the L-3 isozyme exhibited the highest foaming activity and bread-making quality.

The effects of lipoxygenase on the rheological properties of wheat flour doughs have been examined. Daniels et al (1970) proposed that lipid release resulted from a lipoxygenase-mediated coupled oxidation of the sulfhydryl group (SH) and consequent structural changes in the dough protein. Also, Frazier et al (1977) investigated the effects of the soy lipoxygenase enzyme on the mechanical development of wheat flour doughs. They suggested that the the added soy lipoxygenase reacts with the lipids of dough to increase mixing tolerance. Using a mixograph, Hoseney et al (1980) investigated the mechanism by which lipoxygenase increases mixing tolerance. To show the role of lipoxygenase during breadmaking, these investigators used soy flour or partially purified soy lipoxygenase.

In a previous article (Shiiba et al 1990), we suggested that the endogeneous lipoxygenase that is bound with hydrophobic sites of glutenin might be released and activated during fermentation, that the activated lipoxygenase might catalyze oxidation of polyunsaturated fats, and that these products cause changes in the rheological properties of doughs.

To further clarify the role of wheat lipoxygenase during breadmaking, we tried to purify isoenzymes of wheat germ lipoxygenase completely. Then we characterized these purified lipoxygenase isozymes and determined their effects on the baking quality of wheat flour.

MATERIALS AND METHODS

Wheat germ, obtained from No. 1 Canada western red spring wheat, was a flaked product produced by Nisshin Flour Milling Co., Ltd. The bread flour was an unbleached commercial blend. Its protein content was 12.2%, moisture 14.4%, and ash 0.41%. Linoleic and linolenic acid >99% pure substrate were purchased from Sigma Chemical Company, St. Louis, MO. The other reagents used were special grade.

Determination of Lipoxygenase Activity

The linoleic acid emulsion used as the lipoxygenase substrate was prepared by a modification of the method of Surrey (1964). The substrate was prepared in an atmosphere of nitrogen by dissolving 100 μl of pure linoleic acid in a mixture of 0.12 ml Tween 20, 2.5 ml 50-mM acetate buffer (pH 7.0), and 0.32 ml 1.0M sodium hydroxide. After the linoleic acid was dissolved, the mixture was diluted to 50 ml with the 50 mM acetate buffer. The resulting solution was approximately $7.5 \times 10^{-3} M$ in linoleic acid and 0.24% in Tween 20. The substrate was sealed under nitrogen in a syringe bottle and stored at 5°C in the dark before being used as a stock solution. Reaction mixtures consisted of 2.5 ml of phosphate buffer (pH 4.5), 90 μl of stock substrate, and 5 μl of lipoxygenase solution. Lipoxygenase activity at 25°C was assayed spectrophotometrically at 234 nm according to the method of Zimmerman and Vick (1970). A unit of lipoxygenase activity was defined as the amount of enzyme that produces a change of one unit of absorbance at 234 nm per minute.

Estimation of Protein

Protein was determined by the method of Lowry et al (1951) and compared with a standard curve of bovine serum albumin.

Analytical Disk Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Purified lipoxygenase isozymes were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1969), with slight modification. The concentration of acrylamide in the gels was 7.5%, and electrophoresis was done at 3 mA per column (5 × 70 mm) for about 5 hr at 20°C. After electrophoresis, the gel was stained with 0.5% Coomassie Brilliant Blue R250 dissolved in 50% (v/v) methanol containing 7% (v/v) acetic acid for 1 hr at room temperature and destained by washing with gentle shaking four times with a solution of 5% (v/v) methanol containing 7% (v/v) acetic acid. The molecular weight markers used were aldolase (158,000), albumin (68,000), ovalbumin (45,000), and chymotrypsinogen A (25,000), all purchased from Wako Pure Chemical Co., Tokyo.

Amino Acid Analysis

The purified lipoxygenase isozymes were hydrolyzed in evacuated tubes in 6N hydrochloric acid at 110°C for 24 hr. Amino acids were determined using an automatic amino acid analyzer (Hitachi model 835). Tryptophan content was determined by the method of Yamamoto and Hayaishi (1970).

Preparation of the Flour Treated with Purified Lipoxygenase Isozymes

Each purified lipoxygenase isozyme (5,000 units) in 400 ml of 50 mM acetate buffer (pH 5.5) with several drops of toluene was incubated and shaken with the wheat bread flour (500 g) at 40°C for 4 hr. After incubation, the slurry was frozen immediately and lyophilized, ground, and sieved by impact mill (Retch Ultra Centrifugal Mill with a 0.5-mm filter). The control flour was treated the same way but with no enzyme. These flours were used for the mixograph studies and for determining SH groups, SS bonds, and foaming activity.

Determination of Foaming Activity

The foaming activity of flour protein was measured according

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to the modified Kitabatake stirring method (Kitabatake and Doi 1982). Each flour (3.5 g) and 40 ml of 50 m*M* acetate buffer (pH 5.5), in a water-jacketed, 50-ml stainless steel container, were agitated at 4°C in a rotating, six-bladed knife homogenizer (Ace type, Nippon Seiki Kaisha Ltd.). The rotor speed was adjusted to 10,000 rpm. After stirring for 3 min, all of the foam and liquid was immediately transferred to a measuring cylinder by gentle pouring and pipetting, and the volume was measured. Foaming activity (FA) was defined by the expression FA = (F−L)/L × 100. F is the volume of foam plus liquid. L is the volume of the liquid phase of the foam, which is calculated when the weight of foam in the vessel and the density of the liquid are known. *F* and *L* were measured. The FA is dimensionless.

Operation of the Mixograph

Mixograms were obtained in an air-conditioned room maintained at 25°C. The mixograph (National Mfg. Co., Lincoln, NE) was operated at 87 rpm at the No. 9 spring setting (Johnson et al 1946). The flour (30 g, on a 14% moisture basis) was mixed with 20 ml of distilled water.

Determination of SH and SS Contents

SH and SS contents of the flour were determined by amperometric titration using silver nitrate according to the method developed by Sokol et al (1959) and modified by Tsen and Anderson (1963). The results are expressed in micromoles per gram of flour protein.

Bread-Making Test

Using 2,000 g of bread flour and adding 36,000 units of purified lipoxygenase isozymes, breads were baked by the method described by Nagao et al (1981).

RESULTS AND DISCUSSION

Purification of Lipoxygenase Isozymes

To clarify the role of wheat lipoxygenase during breadmaking and to ascertain which lipoxygenase isozymes have a greater effect on bread-making quality, lipoxygenase isozymes were purified from wheat germ. These purified lipoxygenase isozymes then were characterized on the basis of enzymatic properties, amino acid composition, and effects on wheat flour. The isolation and purification procedure for lipoxygenase isozymes at 4°C is summarized in Figure 1.

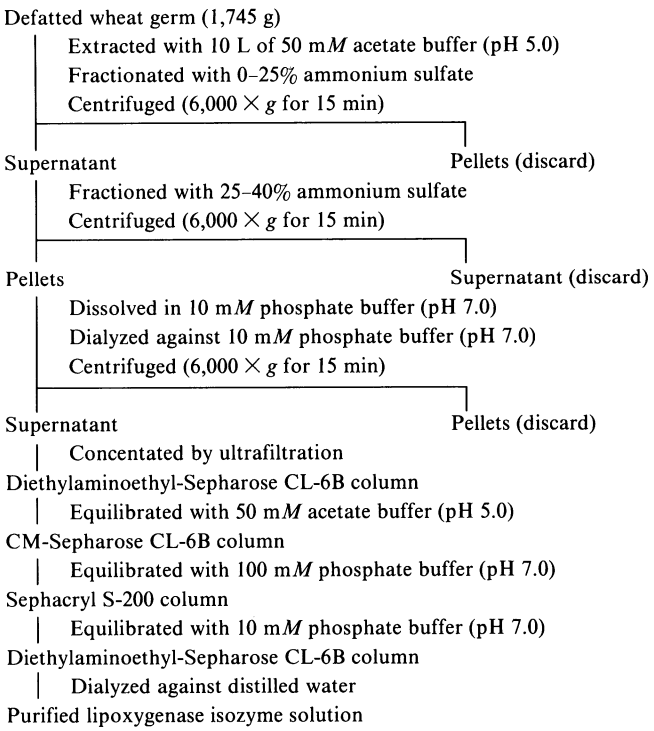

Fig. 1. A scheme for isolation and purification of lipoxygenase isozymes from wheat germ.

Extraction of Lipoxygenase

Fresh wheat germ (2 kg) was defatted by acetone and evaporated to dryness under vacuum. The deffated germ (1,745 g) was then added to 10 L of cold 50 m*M* acetate buffer (pH 5.0). After stirring for 20 min at 4°C, the suspension was centrifuged at 6,000 × *g* for 10 min to obtain a crude enzyme solution.

Ammonium Sulfate Precipitation

After the first precipitation at 25% ammonium sulfate saturation, the mixture was centrifuged at 6,000 × *g* for 15 min. After the second precipitation at 40% saturation, the mixture was allowed to stir for 20 min and then was centrifuged for 15 min.

Diethylaminoethyl-Sepharose CL-6B Chromatography

A Diethylaminoethyl (DEAE)-Sepharose CL-6B column (Pharmacia, 100 × 300 mm) was equilibrated to an effluent pH of 7.0 with 10 m*M* phosphate buffer. After dialyzing against the buffer (pH 7.0), the enzyme solution was applied to the DEAE-Sepharose column. Protein was eluted at pH 7.0 by a linear concentration gradient (0–0.5*M*) of sodium chloride, using a flow rate of 1 ml/min. Eluent was collected in 5-ml fractions and was continuously monitored by light absorption at 280 nm (Fig. 2). Active fractions were pooled and dialyzed against 50 m*M* acetate buffer (pH 5.5) overnight and concentrated further to about 30 ml using an ultrafiltration system (Asahipak C5P, Ashai Kasei Kogyo Co., Tokyo) with a membrane having a molecular weight cutoff of 13,000.

CM-Sepharose CL-6B Chromatography

The enzyme concentrate from the DEAE-Sepharose CL-6B column was loaded on a CM-Sepharose CL-6B column (Pharmacia, 30 × 500 mm) that had been equilibrated with 50 m*M* acetate buffer (pH 5.5). At a flow rate of 0.6 ml/min, a linear concentration gradient of 0–0.5*M* sodium chloride eluted active isozymes to separate into three major isozymes (L-1, L-2, and L-3) and a minor isozyme (L-a) (Fig. 3). The isozymes in 5-ml fractions were pooled and dialyzed against 100 m*M* phosphate buffer (pH 7.0) overnight and concentrated further to about 20 ml, again using ultrafiltration.

Gel Filtration Chromatography

Each isozyme concentrate from the CM-Sepharose CL-6B column was loaded on a Sephacryl S-200 column (Pharmacia, 30 × 1,000 mm) that had been equilibrated with 100 m*M* phosphate buffer (pH 7.0). At a flow rate of 0.4 ml/min, the active

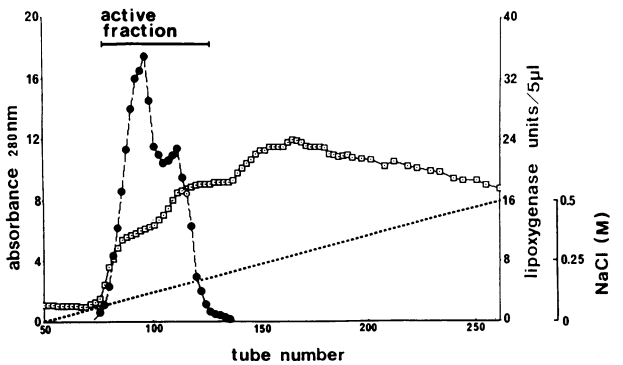

Fig. 2. Separation of wheat germ lipoxygenase by DEAE-Sepharose CL-6B column chromatography at a flow rate of 1 ml/min using 10 m*M* phosphate buffer, pH 7.0, as the eluting buffer with a linear gradient of 0–0.5*M* NaCl. Five-milliliter fractions were collected. ⊡ = absorbance, ● = lipoxygenase, dotted line = NaCl.

isozyme was eluted (Fig. 4). On this column, each peak of lipoxygenase activity was almost coincident with that of the protein. The 5-ml fractions from the column were pooled, dialyzed against 10 m*M* phosphate buffer (pH 7.0) overnight, and concentrated further to about 20 ml using ultrafiltration.

DEAE-Sepharose CL-6B Chromatography

At the final step, each isozyme was loaded on a DEAE-Sepharose CL-6B column (Pharmacia, 10 × 100 mm), as used in the first chromatography step (Fig. 2), which had been equilibrated with 10 m*M* phosphate buffer (ph 7.0). At a flow rate of 0.4 ml/min, a linear concentration gradient of 0–0.2*M* sodium chloride eluted active isozymes in 5-ml fractions (Fig. 5). The fractions were pooled and dialyzed against distilled water; the purified enzyme in solution was stored at 4°C before using.

The purification of isozymes is summarized in Table I. An increase in total activity with ammonium sulfate precipitation suggests that an inhibitor might be removed. Compared with the crude extract, a high degree of purificaton was obtained, but there were different degrees of purification among the isozymes. This might be caused by different properties among the isozymes, especially different isoelectric points. The relationship between isoelectric point differences and catalytic properties among lipoxygenase isozymes is being investigated. Purified protein corresponding to isozyme L-3 exhibited almost 50% of the lipoxygenase isozyme protein, while protein corresponding to isozyme L-a exhibited only 5%.

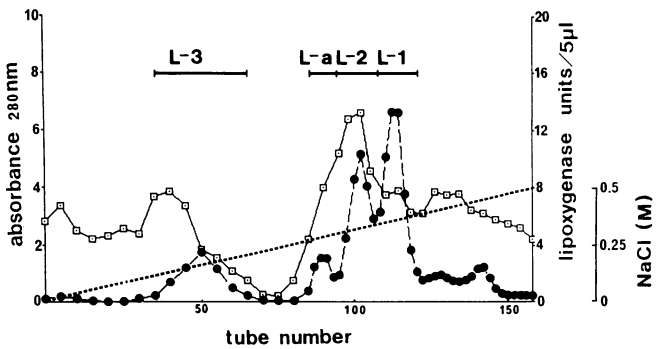

Fig. 3. Separation of wheat germ lipoxygenase isozymes by CM-Sepharose CL-6B column chromatography at a flow rate of 0.6 ml/min using 50 m*M* acetate buffer, pH 5.5, as the eluting buffer with a linear gradient of 0–0.5*M* NaCl. Five-milliliter fractions were collected. Lipoxygenase isozymes were denoted as L-3, L-a, L-2, and L-1, according to their order of elution. ⊡ = absorbance, ● = lipoxygenase, dotted line = NaCl.

Wallace and Wheeler (1979) separated and purified two isozymes of lipoxygenase from wheat germ. However, as Nicolas et al (1982) pointed out, the values of 40,000-fold purification for L-1 and 12,000-fold purification for L-2 seem overestimated. Wallace and Wheeler gave little information about the enzymatic characteristics for these isozymes. Nicolas et al (1982) also reported the purification and some properties of three isozymes from wheat germ. We obtained similar results and also found three major isozymes of lipoxygenase in wheat germ, but we attained a higher degree of purification and separated a minor isozyme (L-a) on the CM-Sepharose CL-6B column.

Analysis of Purified Lipoxygenase Isozymes

SDS-PAGE of lipoxygenase isozymes. SDS-PAGE of the three major lipoxygenase isozymes resulted in single bands with almost the same retardation factor (0.28) (Fig. 6). This provided evidence that each isozyme was purified successfully. On the other hand, it may be that the lipoxygenase isozymes were contaminated by small amounts of other minor proteins that have the same molecular weight and therefore would migrate the same distance. This would affect amino acid analysis. However, isozymes L-1, L-2, and L-3 were well resolved well and separated from each other. from each other, as shown by the correspondence of protein and enzyme peaks (Fig. 5).

Estimating the molecular weight of lipoxygenase isozymes. The molecular weight of each lipoxygenase isozyme was estimated

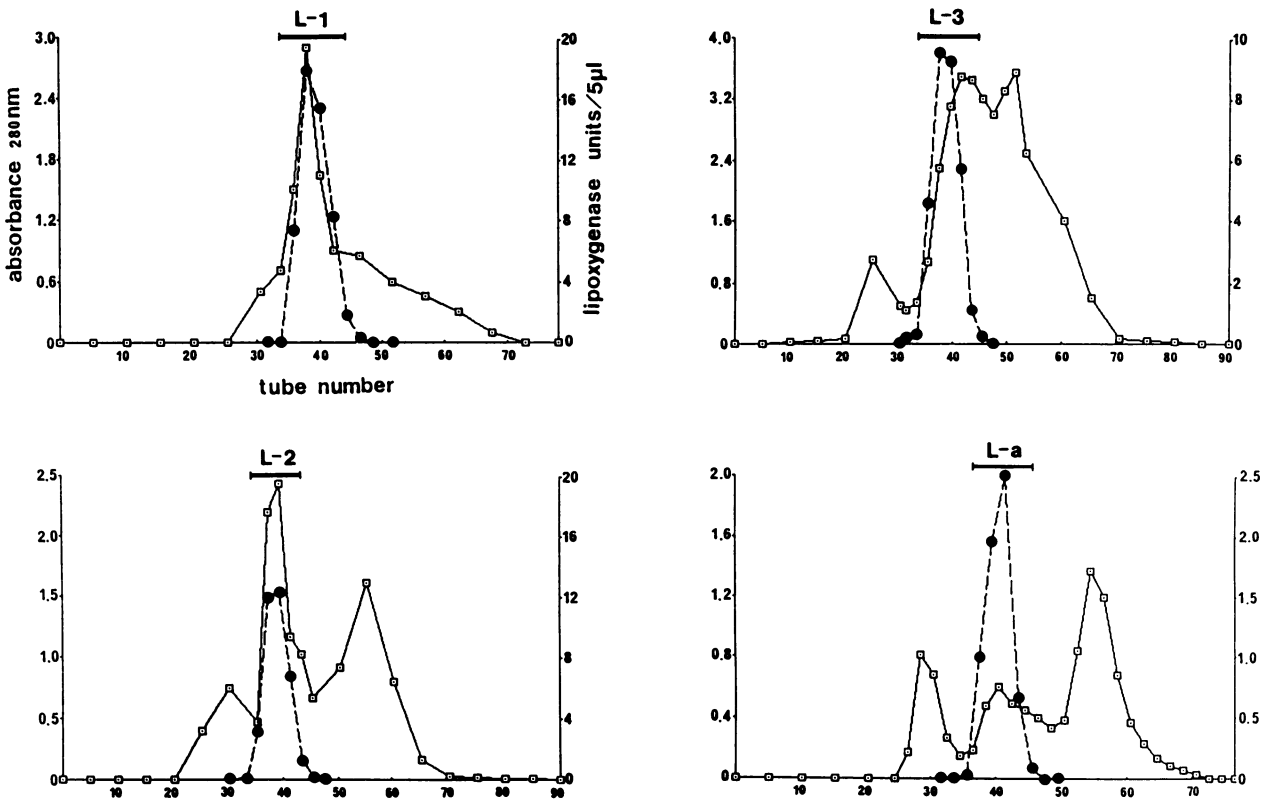

Fig. 4. Elution profile of lipoxygenase isozymes by Sephacryl S-200 column chromatography at a flow rate of 0.4 ml/min using 100 m*M* phosphate buffer, pH 7.0, as the eluting buffer. ⊡= absorbance, ● = lipoxygenase.

by SDS-PAGE using a calibration plot of mobility versus the molecular weight of standard proteins (Fig. 7). A value of about 110,000 was obtained for the three main isozymes (L-1, L-2, and L-3), which was higher than the molecular weights ranging from 84,000 to 89,000 obtained by Wallace and Wheeler (1979) but the same as those reported by Nicolas et al (1982) using electrophoresis in an acrylamide gradient. The obtained values are also close to other results reported for lipoxygenases isolated from soybean (Mitsuda et al 1967, Diel and Stan 1978).

Profile of pH activity. All lipoxygenase isozymes exhibited similar pH activity profiles (Fig. 8), although the L-3 isozyme showed higher activity over a wider pH range (optimum pH, 4.5–6.0). The optimum pH of L-1 and L-2 isozymes was 5.5. Nicolas et al (1982) and Wallace and Wheeler (1979) reported an optimum pH of 6.0–6.5 for all lipoxygenase isozymes. By comparison, the pH optimums for the lipoxygenase isozymes purified in this study were lower. Hsieh and McDonald (1984) indicated similar values for lipoxygenase isozymes partially purified from durum wheat.

Thermal sensitivity. The three major lipoxygenase isozymes had

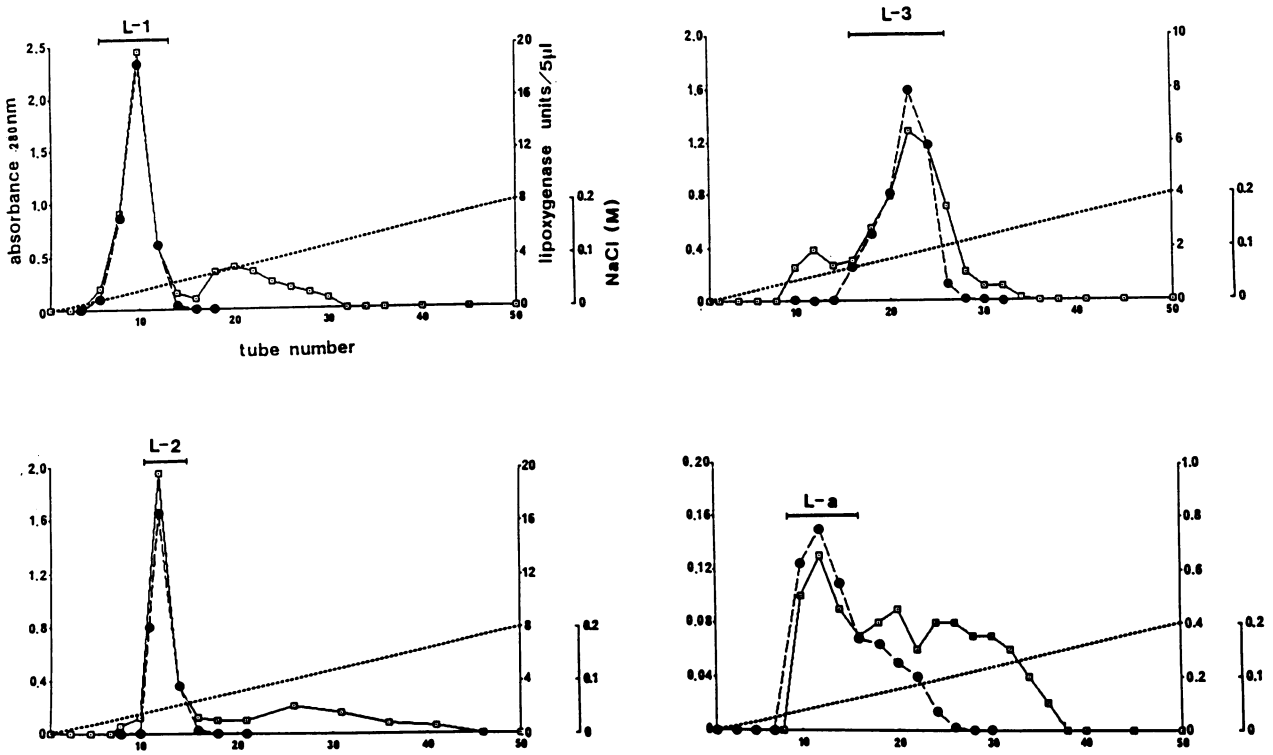

Fig. 5. Elution profile of lipoxygenase isozymes by DEAE-Sepharose CL-6B column chromatography at a flow rate of 0.4 ml/min using 10 m*M* phosphate buffer, pH 7.0, as the eluting buffer with a linear gradient of 00–0.2*M* NaCl. ⊡ = absorbance, ● = lipoxygenase, dotted line = NaCl.

TABLE I
Purification Steps of Wheat Germ Lipoxygenase Isozymes

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Degree of Purification
Crude extract	162,572	469,833	2.89	100	1
$(NH_4)_2$, SO_4, 0.25–0.4 saturation	31,428	494,991	15.75	105.4	5.4
Diethylaminoethyl-Sepharose	3,136	337,360	107.6	71.8	37.2
CM-Sepharose	971.5	203,836	209.8	43.4	72.6
L-1	159.0	81,232	510.9	17.3	176.8
L-2	164.0	51,184	312.1	10.9	108.0
L-3	524.2	58,294	111.2	12.4	38.5
L-a	124.3	13,126	105.6	2.8	36.5
Sephacryl S-200	274.4	176,014	641.5	37.5	222.0
L-1	73.7	78,085	1,059.5	16.6	366.6
L-2	48.5	49,417	1,018.9	10.5	352.6
L-3	129.6	37,533	289.6	8.0	100.2
L-a	22.6	10,979	485.8	2.3	168.1
Diethylaminoethyl-Sepharose	103.8	121,368	1,069.2	25.8	370.0
L-1	27.9	51,492	1,845.6	11.0	638.6
L-2	18.4	27,830	1,512.5	5.9	523.4
L-3	52.4	36,357	693.3	7.7	239.9
L-a	5.1	5,689	1,111.1	1.2	384.5

almost the same thermal sensitivity, with optimum activity at around 45° C and only traces of activity at 65° C (Fig. 9).

Comparison of activity toward linoleic and linolenic acid. Comparison of two substrates (linoleic and linolenic acid) showed that activity toward linoleic and linolenic acid is the better substrate for all lipoxygenase isozymes. L-3 and L-a oxidized linolenic acid with a relative activities of 83% and 77%, respectively, compared with their activity toward linoleic acid, but L-2 showed only 7%, and L-1 28%. Truong and Mendoza (1982), however, reported that of two lipoxygenase isozymes (L-1 and L-2) from cowpea, only the L-2 isozyme responded to linolenic acid with a relative activity of 45% compared with its activity toward linoleic acid.

Effect of metal ions. The inhibitory effect of metal ions (0.001 *M*) on lipoxygenase isozymes is shown in Table II. Cu^{2+} and Hg^{2+} showed strong inhibitory effects. Mn^{2+} and Fe^{2+} showed a weak inhibitory effect. Each isozyme had different responses to metal ions, with more inhibition observed in general for isozymes L-1 and L-2 than for L-3 and L-a. This result was similar to that observed for lipoxygenase isozymes from different sources (Yamamoto et al 1970, Shastry and Rao 1975, Truong and Mendoza 1982).

Amino acid composition. As shown in Table III, the relatively high levels of aspartic acid, glutamic acid, glycine, and leucine

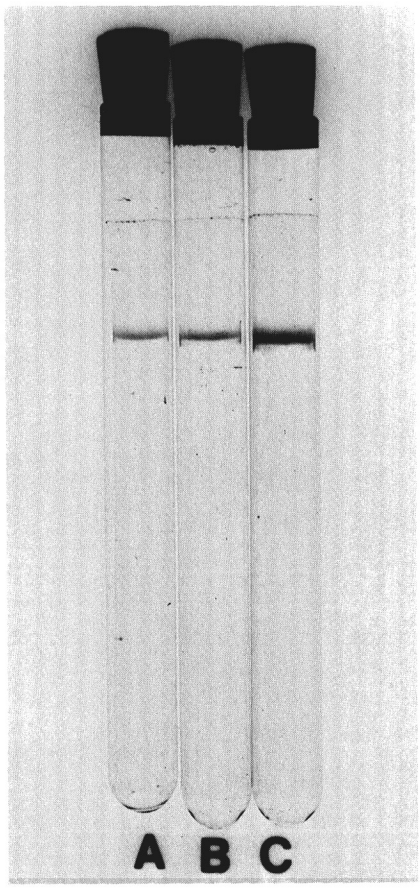

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of purified wheat germ lipoxygenase isozymes. **A,** L-1; **B,** L-2; **C,** L-3.

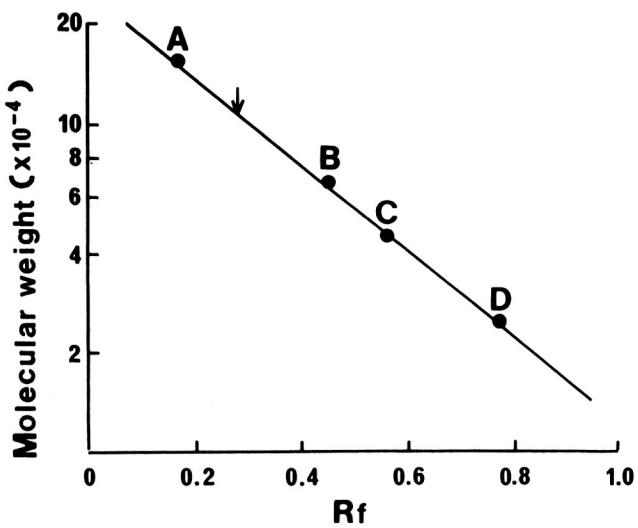

Fig. 7. Estimation of the molecular weight of lipoxygenase isozymes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The weight markers used were: A, aldolase (158,000); B, alubumin (68,000); C, ovalbumin (45,000); D, chymotrypsinogen A (25,000). The arrow indicates the retardation factor of the lipoxygenase isozymes.

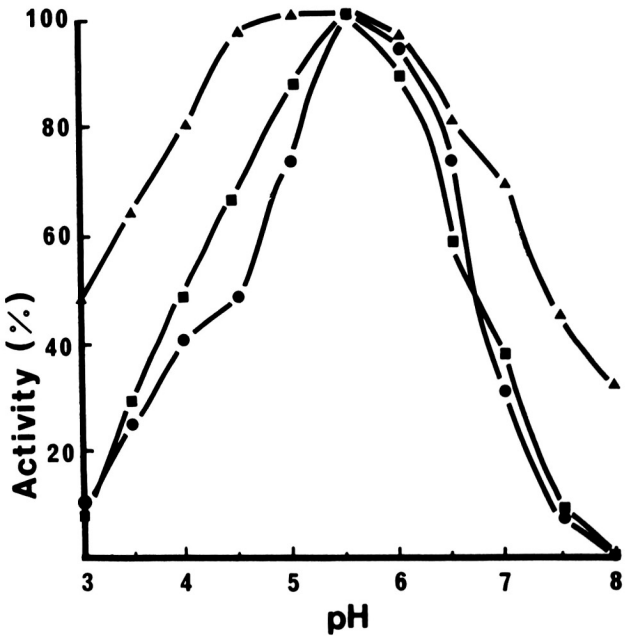

Fig. 8. Effect of pH on the enzyme activity of purified wheat germ lipoxygenase isozymes. ● = L-1; ▲ = L-2; ■ = L-3.

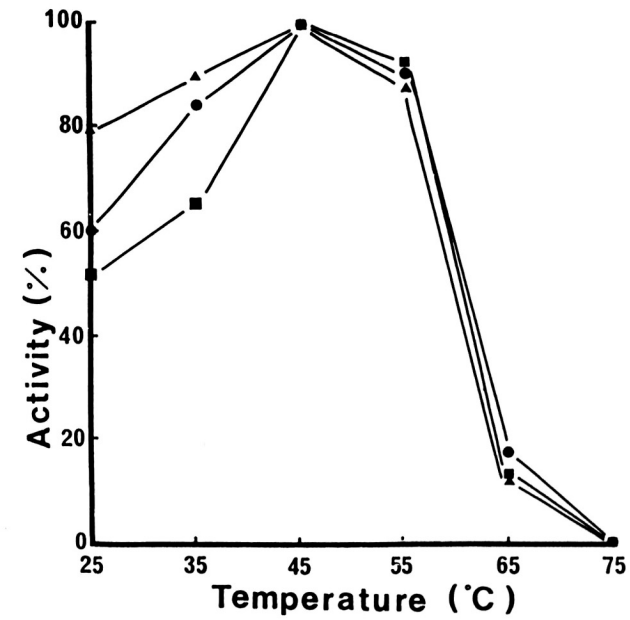

Fig. 9. Thermal sensitivity of purified wheat germ lipoxygenase isozymes. ● = L-1; ▲ = L-2; ■ = L-3.

were characteristic of all purified lipoxygenase isozymes, as is shown in other reports (Eriksson and Svensson 1970, Stevens et al 1970, N'Doye et al 1985). However, the L-3 isozyme was somewhat different from other isozymes because it had higher amounts of serine, glutamic acid, and glycine and lower amounts of methionine, tyrosine, and lysine. Amino acid compositions of L-1 and L-2 correlated considerably better ($r = 0.996$) than those of L-1 and L-2 ($r = 0.93$) or L-2 and L-3 ($r = 0.88$). However, no statistically significant differences were evident among the three isozymes.

Effect of Purified Lipoxygenase Isozymes on Wheat Flour

Determination of foaming activity. The flours treated with purified lipoxygenase isozymes exhibited higher foaming activity than did the control flour without enzyme treatment. Of the flours treated with enzyme, the one treated with the L-3 isozyme had especially higher foaming activity (53.4) than that of the control flour (42.9). The flours treated with the L-1 and L-2 isozymes showed slightly higher foaming activity (44.4 and 46.7, respectively). The relationship between foaming activity and lipoxygenase activity is significant because it might influence rheological properties. For example, in a previous article, Shiiba et al (1990) indicated that lipoxygenase activity in fermented dough might be related to the hydrophobicity of acetic acid-soluble proteins. Those results suggested that rheological changes during fermentation are due to a decrease in the surface hydrophobicity of soluble glutenin, induced by the binding of oxidation products of lipoxygenase.

Mixograms of flours treated with purified lipoxygenase isozymes. As shown in Figure 10, mixograms of the flours treated with a lipoxygenase isozyme (especially L-3), exhibited shorter dough development time and less resistance after maximum consistency (peak time), than did the control flour. In the same study, Shiiba et al (1990) showed that as fermentation time was increased, fermented dough showed less resistance to extension and a shorter peak time, concomitant with increased lipoxygenase activity. The

TABLE II
Effect of Different Metal Ions on the Activity of Wheat Germ Lipoxygenase Isozymes[a]

Metal Compounds (1 m*M*)	Relative Activity, %			
	L-1	L-2	L-3	L-a
None	100	100	100	100
$CaCl_2$	100	115	104	112
$MgCl_2$	96	130	100	101
$ZnCl_2$	101	114	98	95
$HgCl_2$	0	0	0	0
$MnCl_2$	50	50	88	92
$BaCl_2$	93	84	104	115
$CuCl_2$	2	5	33	20
$FeCl_2$	70	50	90	89

[a] Values are the average of two replications. All duplicates were within 5% of the mean.

TABLE III
Amino Acids Composition of Wheat Germ Lipoxygenase Isozymes[a]

Amino Acid	Mole Percent			
	L-1	L-2	L-3	L-a
Aspartic acid	9.8	10.1	9.6	11.8
Threonine	5.6	5.7	5.4	5.4
Serine	6.9	6.2	10.3	6.7
Glutamic acid	10.1	9.3	13.2	9.3
Proline	9.0	7.9	7.8	9.4
Glycine	10.0	9.6	13.0	11.1
Alanine	7.7	7.8	7.9	8.8
Cystine	0.6	0.4	0.7	0.3
Valine	6.4	6.6	5.6	4.4
Methionine	1.0	1.6	0.5	1.0
Isoleucine	3.9	4.2	3.4	3.1
Leucine	9.2	10.1	7.3	9.3
Tyrosine	3.3	3.5	1.8	3.2
Phenylalanine	3.4	3.2	2.8	3.1
Lysine	4.6	4.9	3.4	4.6
Histidine	3.2	3.7	3.0	2.8
Arginine	5.2	5.1	4.3	4.7
Tryptophan	0	0	0	0

[a] Values are the average of two replications. All duplicates were within 5% of the mean.

TABLE IV
Effect of Wheat Germ Lipoxygenase Isozymes on Sulfhydryl (SH) and Disulfide (SS) Contents[a]

Lipoxygenase Isozyme	SH (μeq/g of protein)	SS (μeq/g of protein)	SH/SS (%)
Control	16.45[b]	101.93[b]	16.1[b]
L-1	15.47	106.79	14.5
L-2	15.63	110.09	14.2
L-3	15.41	106.60	14.5

[a] Values are the average of two replications. All duplicates were within 5% of the mean.
[b] Significant difference ($P < 0.01$) between the marked value and others.

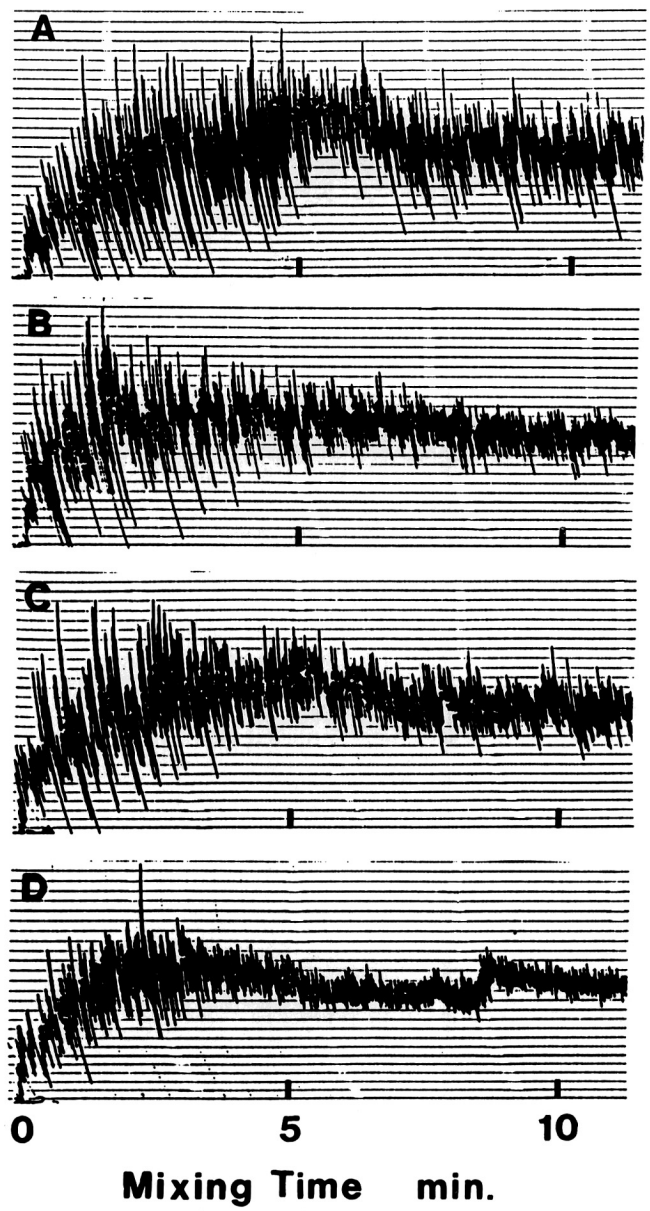

Fig. 10. Mixograms showing effects of purified wheat germ lipoxygenase isozymes on supplemented flour. **A,** control flour without enzyme supplement; **B, C,** and **D,** flours supplemented with isozymes L-1, L-2, and L-3, respectively.

TABLE V
Effect of Purified Wheat Germ Lipoxygenase Isozymes on Baking Performance[a]

Flour Dough	Loaf Volume (cc)	Relative Loaf Volume
Control	1,800	100
Treated with Isoenzyme		
L-1	1,840	102
L-2	1,860	103
L-3	1,980[b]	110

[a] Values are the average of two replications. All duplicates were within 5% of the mean.
[b] Highly significant difference ($P < 0.001$) between the marked value and others.

results of the present study suggested that purified lipoxygenase caused caused a similar mixogram performance. Compared with soybean lipoxygenase (Hoseney et al 1980), wheat lipoxygenase might affect the physical properties of the dough differently than soy lipoxygenase does since adding soy flour lipoxygenase to wheat flour increased the mixing tolerance. In particular, the effect of adding the L-3 isozyme to the flour especially might be similar but less pronounced than that of fast-acting oxidants such as potassium iodate.

It is not clear why the flours treated with lipoxygenase isozymes exhibited different mixograph properties compared with flours supplemented with soy lipoxygenase. One possible reason is that the oxidation mechanism for soybean may be different from that for wheat germ lipoxygenase. Soybean lipoxygenase attacks unsaturated triglycerides, whereas wheat lipoxygenase mainly reacts with free fatty acid (Veldink et al 1977). Alternatively, each lipoxygenase isozyme might behave differently at its reactive sites (Waller and Wheeler 1979). Further work is required to explore this oxidation mechanism.

Effect of lipoxygenase on SH group and SS bond content in treated doughs. The flours supplemented with each purified lipoxygenase isozyme exhibited lower contents of SH groups and higher contents of SS bonds than did the control flour (Table IV). An increase in SS content of the treated flour apparently could be affected by lipoxygenase-mediated oxidation of SH groups in flour protein. The SH content did not differ among the flours treated with the three main lipoxygenase isozymes. Some investigators (Koch 1956, Tsen and Hlynka 1963) have suggested that the loss of SH groups when soy flour lipoxygenase was added, caused by enzyme-active soy flour, was due to oxidation of SH groups. The present study agrees closely with these reports. However, additional work is necessary for comparison.

Bread-making test. The bread made from the flour treated with the L-3 isozyme had the highest volume (Table V). The others had almost the same volume as the control, but their crumb and crust were whiter. This result supports the conclusion that bleaching of carotenoids is due to lipoxygenase activity (Barrett 1975, McDonald 1979). Improvement in bread-making properties paralleled the changes in foaming activity in the flours treated with the lipoxygenase isozymes. This result indicates that each lipoxygenase isozyme might play a differential role in breadmaking. The L-3 isozyme, especially, played a significant role in influencing bread-making quality.

CONCLUSION

After extraction from defatted wheat germ with acetate buffer (pH 5.0) and precipitation with ammonium sulfate (25–40%), three major lipoxygenase isozymes (L-1, L-2, and L-3) and one minor isozyme (L-a) were separated and purified by DEAE-Sepharose CL-6B and CM-Sepharose CL-6B and by gel-filtration using Sephacryl S-200 column chromatography. Purified protein corresponding to the L-3 isozyme exhibited almost 50% of total lipoxygenase isozyme protein purified by DEAE-Sepharose CL-6B column chromatography. SDS-PAGE of the three major lipoxygenase isozymes resulted in single bands with almost the same retardation factor (0.28). The molecular weight of the lipoxygenase isozymes, obtained by SDS-PAGE, was approximately 110,000, which confirms the results reported by Nicolas et al (1982).

Differences in chemical and physical properties show that the isozymes had different enzymatic properties. In particular, the L-3 isozyme oxidized linolenic acid with high activity, while the L-1 and L-2 isozymes showed weak or no activity in this regard.

Flours supplemented with purified lipoxygenase isozymes exhibited higher foaming activity, shorter dough development time, less resistance after peak time on mixogram, and lower SH contents than did the control flour. The flour supplemented with the L-3 isozyme exhibited the highest foaming activity and the greatest influence on bread-making quality. The present study suggests that the L-3 isozyme plays a significant role in bread-making.

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