Isolation of Lipoxygenase Isoenzymes from Flour of Durum Wheat Endosperm[1]

C. C. HSIEH and C. E. McDONALD,[2] Department of Cereal Chemistry and Technology, North Dakota State University, Fargo 58105

Cereal Chem. 61(5): 392–398

ABSTRACT

Three lipoxygenase isoenzymes from flour of durum wheat endosperm were isolated and partially purified by ammonium sulfate fractionation and by DEAE- and CM-cellulose column chromatography. The three isoenzymes are designated as lipoxygenase-1 (L-1), lipoxygenase-2 (L-2), and lipoxygenase-3 (L-3), according to their inverse order of elution from CM-cellulose column. The L-3 isoenzyme (first off the column) and the L-2 isoenzyme (next off the column) had optimum lipoxygenase activity at pH 4.8, and the L-1 (last off the column) had activity optima at pH 10.2 and 11.4. The L-1 isoenzyme also had peroxidase activity with an optimum at pH 4.8. The L-1 was further fractionated by preparative disk polyacrylamide gel electrophoresis. Agarose-con A affinity chromatography of L-1 gave three active fractions, all having peroxidase, lipoxygenase, and carotene bleaching activity. Both L-2 and L-3 isoenzymes exhibited optimum carotene bleaching activity at pH 6.6, whereas L-1 had its optimum at pH 10.2. All three isoenzymes required high concentration of linoleic acid for carotene bleaching, and the L-1 isoenzyme also required high linoleic acid concentration for linoleic acid oxidation. The relative lipoxygenase activity in crude extract from endosperm flour of Golden Ball durum wheat was 21.3, 33.8, and 44.9%, respectively, for L-1, L-2, and L-3 at their pH of optimum activity.

The bright yellow color of pasta products is attributed to the natural carotenoid pigments of amber durum wheat. This yellow color is usually one of the major criteria of quality (Irvine 1971). Macaroni with a high content of yellow pigment can be obtained only from durum semolina that is high in carotenoid pigments. This factor in itself, however, does not necessarily ensure a good yellow pasta product, because of the oxidative degradation of yellow pigments by lipoxygenase to colorless or off-color pigments during processing of pasta products (Irvine and Winkler 1950).

Lipoxygenase (EC 1.13.11.12) of durum wheat semolina has been studied manometrically (Irvine and Anderson 1953a, 1953b; Irvine 1959) and spectrophotometrically (Walsh et al 1970, McDonald 1979). Among the varieties examined, Golden Ball was ranked highest in lipoxygenase activity followed by Pelissier (Irvine and Anderson 1953b, Walsh et al 1970, McDonald 1979).

Activity optima (pH optima) reported for lipoxygenase of durum wheat semolina are pH 4.8 (McDonald 1979), pH 5.9 (Walsh et al 1970), and pH 6.5 (Irvine and Anderson 1953a). McDonald (1979) also observed peaks of activity at pH 6.0 and 7.0. An activity optimum of pH 9.0 was found by McDonald (1979) for the bleaching of the carotenoid lutein by enzymes of semolina, but there also were two smaller peaks of activity at pH 6.0 and 4.0. The peaks of activity occurring over a range of pH suggests that more than one enzyme (isoenzymes) has lipoxygenase and carotenoid bleaching activity in durum wheat endosperm. Several lipoxygenase isoenzymes from soybeans have been isolated and extensively studied (Axelrod 1974, Axelrod et al 1981), but the isolation and purification of endogenous lipoxygenase from flour of durum wheat endosperm has not been reported. This paper describes the isolation and purification of durum wheat lipoxygenase.

MATERIALS AND METHODS

Materials

Golden Ball variety of durum wheat (14.8% protein content, 14% moisture basis) was grown in 1979 at Langdon, ND. Wheat was cleaned, tempered to 17.5% moisture, and milled on a Brabender Quadruplex (C. W. Brabender Instruments, Inc., South Hackensack, NJ) to give 53.5% yield of flour (12.7% protein, 14% mb). This flour was stored in a sealed container at 4° C. Moisture and protein in wheat and flour were determined by AACC methods 44-11, 44-15A, and 46-10 (AACC 1982). Chemicals used throughout this study were analytical grade unless otherwise stated.

Enzyme Substrate Preparation and Enzyme Assay

Lipoxygenase. The linoleic acid emulsion used as the lipoxygenase substrate was prepared by the method of Surrey (1964), with modifications similar to that of Ben Aziz et al (1970). The concentrations of linoleic acid (99%+, Nu Check Prep, Elysian, MN) and Tween 20 were $8.02 \times 10^{-3} M$ and 2.5 μl/ml, respectively, in 0.05*M* sodium borate buffer (pH 9) prepared with deoxygenated solvents and stored at −15° C under N_2.

For lipoxygenase assays at high linoleic acid concentration and for bleaching assays, the substrate emulsion was prepared by the method of Ben Aziz et al (1971). In a second high concentration, linoleic acid substrate Tween 80 was replaced by Tween 20, and the ethylenediamine tetraacetic acid (EDTA) was omitted. The concentrations of linoleic acid, Tween 80, EDTA, and Tween 20 were $2.67 \times 10^{-2} M$, 3.2 μl/ml, 0.25%, and 1.6 μl/ml, respectively. The substrates were also stored at −15° C under N_2.

Lipoxygenase activity at 25° C was determined by measuring conjugated diene absorption at 234 nm by the procedure described by Zimmerman and Vick (1970) in a Beckman DB-G spectrophotometer fitted with a constant-temperature water-jacketed cell holder and a strip-chart recorder. For low concentration of linoleic acid, the reaction mixture in 3-ml volume contained 25° C nondeoxygenated 0.05*M* buffer of desired pH, enzyme extract and 0.025 ml of linoleic acid substrate solution ($8.02 \times 10^{-3} M$), which were added to the cell in the sequence given. For lipoxygenase assay at high concentration of linoleic acid, 0.15 ml of Tween 80 solution (3.2 μl/ml) or Tween 20 solution (4.8 μl/ml), 0.15 ml of linoleic acid ($2.67 \times 10^{-2} M$), 1.68 ml of buffer of desired pH, and 0.02 ml of enzyme were added to the cell in the order given. At low substrate concentration, the initial linoleic acid and Tween concentration were $6.7 \times 10^{-5} M$ and 0.02 μl/ml, respectively; at high substrate concentration they were $2 \times 10^{-3} M$ and 0.48 μl/ml, respectively. One unit of enzyme activity corresponded to the production of 1 μmole of conjugated hydroperoxydienoic per minute, using a molar absorptivity of 2.8×10^4 (Privett et al 1955).

Carotene Bleaching

This activity at 25° C was determined by measuring the decrease in absorbance at 460 nm according to the method of Ben Aziz et al (1971) with some modifications. The final reaction mixture of 2 ml of volume contained 0.15 ml of β-carotene ($55.8 \times 10^{-6} M$), 0.15 ml of linoleic acid ($2.67 \times 10^{-2} M$), and buffer (0.05*M*) of desired pH added to the cell in the sequence given. The solution was mixed

[1]Published with approval of the Director of the North Dakota Agricultural Experiment Station as Journal Series No.1318.

[2]Project research assistant and professor, respectively. Present address of C. C. Hsieh: USC Cancer Research Center, CRL 104, School of Medicine, University of Southern California, 1303 N. Mission Rd., Los Angeles, CA 90033.

rapidly, and enzyme preparation (0.02 or 0.08 ml) was added to initiate the reaction. One unit of activity corresponded to the destruction of one μmole of β-carotene per minute. μMoles of β-carotene were calculated using a molar absorptivity of 1.235×10^5 or a standard calibration curve of aqueous β-carotene-linoleate mixture. The concentration of reagents in the initial reaction mixture were as follows: β-carotene, $4.2 \times 10^{-6} M$; linoleic acid, $2.0 \times 10^{-3} M$; EDTA, $1.0 \times 10^{-3} M$; and Tween 80 or 20, 0.48 μl/ml.

Peroxidase

The peroxidase activity at 25° C was assayed by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol to reddish brown tetraguaiacol in the presence of H_2O_2 (Chance and Maehly 1955). The assay mixture contained 2.80 ml of $0.05 M$ buffer of desired pH, 0.05 ml of $2.0 \times 10^{-2} M$ guaiacol dissolved in water, and 0.1 ml of enzyme preparation. Also $5 \times 10^{-4} M$ Ca++ was added for activating L-1 peroxidase in later assays. The assay was initiated by addition of 0.05 ml of $8.0 \times 10^{-3} M$ H_2O_2 (A235 nm ≅ 0.45, equivalent to the absorption reported by Chance 1954). One unit of peroxidase activity corresponded to one μmole of H_2O_2 consumed per minute in the assay. μMoles H_2O_2 were calculated using a molar absorptivity of 2.66×10^4 for tetraguaiacol (Maehly and Chance 1954) and 0.25 μmoles of tetraguaiacol being produced from 1 μmole of H_2O_2.

All assays on enzyme preparations were performed in duplicate or triplicate.

Isolation of Lipoxygenase and Carotene Bleaching Enzyme

The isolation and purification of lipoxygenase isoenzymes at 2–5° C is summarized in Fig. 1. After the first precipitation at 30% saturation with ammonium sulfate, the mixture was centrifuged at 18,000 × *g* for 30 min and after the second precipitation at 60% saturation, following standing overnight, the centrifugation was for 70 min.

For chromatography DEAE-cellulose (DEAE-32, Whatman, Inc., Clifton, NJ) was treated before use and packed by the method described in the manufacturer's information leaflet. The packed column (4.4 × 50 cm) was then equilibrated to an effluent pH of 6.8 with $0.05 M$ sodium phosphate buffer. After dialyzing against three 10-fold volumes of $0.05 M$ sodium phosphate buffer (pH 6.8), the enzyme fraction (75 ml) containing 2,025 mg of protein was applied to the DEAE-cellulose column. Protein was eluted at pH 6.8 by a linear concentration gradient from 0.05 to $0.18 M$ sodium phosphate buffer, using a flow rate of 40 ml/hr and collected in 10-ml fractions while the eluant was continuously monitored by light absorption at 280 nm (model 226 Absorption Monitor, ISCO, Lincoln, NE). Active fractions from two consecutive runs were pooled and concentrated in dialysis bags by covering with sucrose. The concentrate was simultaneously dialyzed against $0.05 M$ sodium citrate buffer (pH 5) while concentrated further to about 38.1 ml in a dialysis concentrator (Micro-ProDicon Unit, Bio-Molecular Dynamic, Beaverton, OR), with a membrane having a molecular-weight cutoff of 15,000.

Protein in the concentrate was determined by the method of Lowry et al (1951) and compared to a standard curve on bovine serum albumin prepared by plotting reciprocal absorbance (A^{-1}) versus reciprocal protein concentration (P^{-1}), as suggested by Coakley and James (1978).

The enzyme concentrate (2,780 mg of protein) from the DEAE-cellulose column was loaded on a CM-cellulose column (CM-32, Whatman, Inc.) (4.4 × 50 cm) that had been equilibrated with $0.05 M$ sodium citrate buffer at pH 5.0. At a flow rate of 15 ml/hr, a linear concentration gradient of $0.05–0.7 M$ in pH 5.0 sodium citrate buffer eluted active isoenzyme, and the isoenzymes in 10-ml fractions were pooled and concentrated by sucrose as already described.

The last isoenzyme (L-1) was further concentrated by a dialysis concentrator against $0.035 M$ β-alamine-acetate buffer at pH 4.5, in preparation for preparative disk polyacrylamide electrophoresis by a method adapted from the analytical procedure described by Reisfeld et al (1962). Using a Buchler apparatus (Poly Prep 100, Buchler Instruments, Inc., Fort Lee, NJ), the sample (2 ml, 100 mg of protein) was applied carefully in 10% glycerol containing 0.01 mg of methyl green per milliliter as a layer on the surface of the stacking gel. During electrophoresis at 4° C, a constant current of 30 mA was used for 3 hr, and then it was raised to 50 mA throughout the rest of the run. Proteins migrating off the end of the column were eluted with $0.045 M$ potassium acetate buffer (pH 4.3) at a flow rate of 0.5 ml/min and collected in 4-ml fractions. The enzyme-active fractions from four consecutive runs were pooled for further studies.

Analytical Disk Polyacrylamide Gel Electrophoresis

Isolated isoenzymes (L-2 and L-3) were examined by disk electrophoresis (Polyanalyst Apparatus; Buchler Instruments, Inc., Fort Lee, NJ) according to the method of Reisfeld et al (1962) at acidic pH and the method of Davis (1964) at basic pH. Gels were fixed in 20% trichloroacetic acid (TCA) for 5 min and then stained with 0.5% serva blue W (Fine Chemicals, Inc., Garden City, NY) in water for 5 min. The gels were destained with several changes of water and stored in water.

Carbohydrate Affinity Chromatography

Glycosylex-A (concanavalin-A attached to agarose) (Miles Laboratories, Elkhart, IN), was used to separate glycoproteins in the L-1 enzyme in a procedure similar to the method described by Kahn et al (1981). A 0.9 × 8-cm glass column was packed with Glycosylex-A resin equilibrated with $0.01 M$ sodium phosphate buffer (pH 7.0) containing $0.15 M$ NaCl. An aliquot (1.15 ml) of electrophoretically purified L-1 enzyme concentrate (15 mg of protein) in $0.01 M$ sodium phosphate buffer (pH 7.0) containing $0.15 M$ NaCl was added to the column, and the enzyme and adsorbent were allowed to equilibrate for 1 hr at 4° C. Unadsorbed material was eluted with 30 ml of $0.01 M$ sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min and then adsorbed material with 40 ml of the same buffer containing 5% 2-methyl-D-mannoside. Fractions (2 ml) were assayed for bleaching and peroxidase activities.

RESULTS AND DISCUSSION

Isolation of Lipoxygenase Isoenzymes

Figure 1 outlines the isolation procedure. During the isolation, bleaching activity was assayed at pH 9.2, lipoxygenase at pH 4.8, and peroxidase at pH 7.0. A previous study (McDonald 1979) and our preliminary work indicated that, at pH 4.8, lipoxygenase activity is high and bleaching activity is low, whereas at pH 9.2, bleaching activity is high and lipoxygenase activity is low. Preliminary studies also indicated that more pH 9.2 carotene bleaching activity was extracted with $0.05 M$ sodium citrate at pH

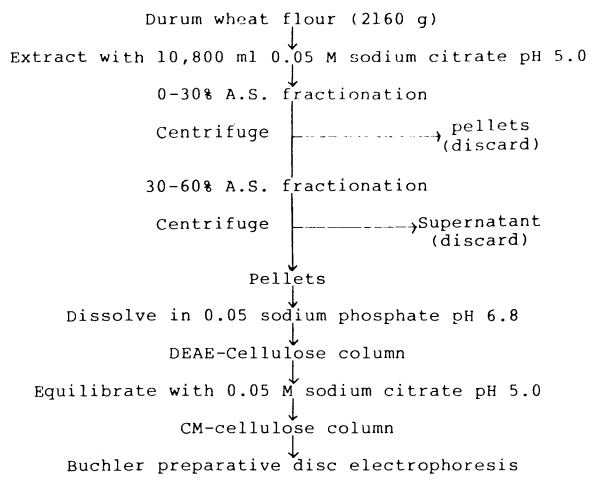

Fig. 1. A scheme for isolation and purification of lipoxygenase isoenzymes from flour of durum wheat endosperm. A.S. = ammonium sulfate.

5.0 than with 0.1 *M* sodium phosphate at pH 7.0, but about equal amounts of pH 4.8 lipoxygenase activity was extracted with both buffers. Also most lipoxygenase and carotene bleaching activity precipitated between 30 and 60% saturation with ammonium sulfate.

On a DEAE-cellulose column, both lipoxygenase and carotene bleaching activity eluted in the fast-running protein fraction (Fig. 2), indicative of low net negative charge at pH 6.8. Although lipoxygenase activity was only partially separated from carotene bleaching activity, the latter activity overlapped almost identically with pH 7.0 peroxidase activity. Active fractions from two consecutive runs were pooled. After being dialyzed and concentrated, the enzyme material separated on a CM-cellulose column into two lipoxygenase peaks (L-3 and L-2) and one carotene bleaching peak (L-1) (Fig. 3). These three peaks of activity were later found to possess both lipoxygenase and carotene bleaching activity and are designated as lipoxygenase-3 (L-3), lipoxygenase-2 (L-2), and lipoxygenase-1 (L-1), according to their inverse order of elution. Peroxidase separated into two active peaks, one associated with carotene bleaching peak (L-1). The elution profile also idicated that L-1 is the most basic lipoxygenase and, therefore, the most highly adsorbed. The next to the most weakly bound was L-2, and the weakest bound was L-3.

The L-2 and L-3 from the CM-cellulose column (Fig. 3) may be similar to the two major lipoxygenase fractions isolated from wheat germ by Wallace and Wheeler (1979) and Nicolas et al (1982) and from wheat grain (*Triticum aestivum* L.), by Weber et al (1973). L-1 isoenzyme migrated mostly as one peak by preparative disk electrophoresis with both carotene bleaching and peroxidase activity (Fig. 4). In red pepper fruits, Kanner et al (1977) were also unable to separate the carotene bleaching factor from peroxidase. Since both activities had the same pH optimum, they concluded the peroxidase was indeed responsible for the bleaching of β-carotene. However, here the pH optimum for peroxidase is 4.8 and for the carotene bleaching activity 10.2.

The purification of isoenzymes is summarized in Tables I and II. An increase in total activity of L-1 with DEAE-cellulose chromatography (Table I) indicates that an inhibitor was removed.

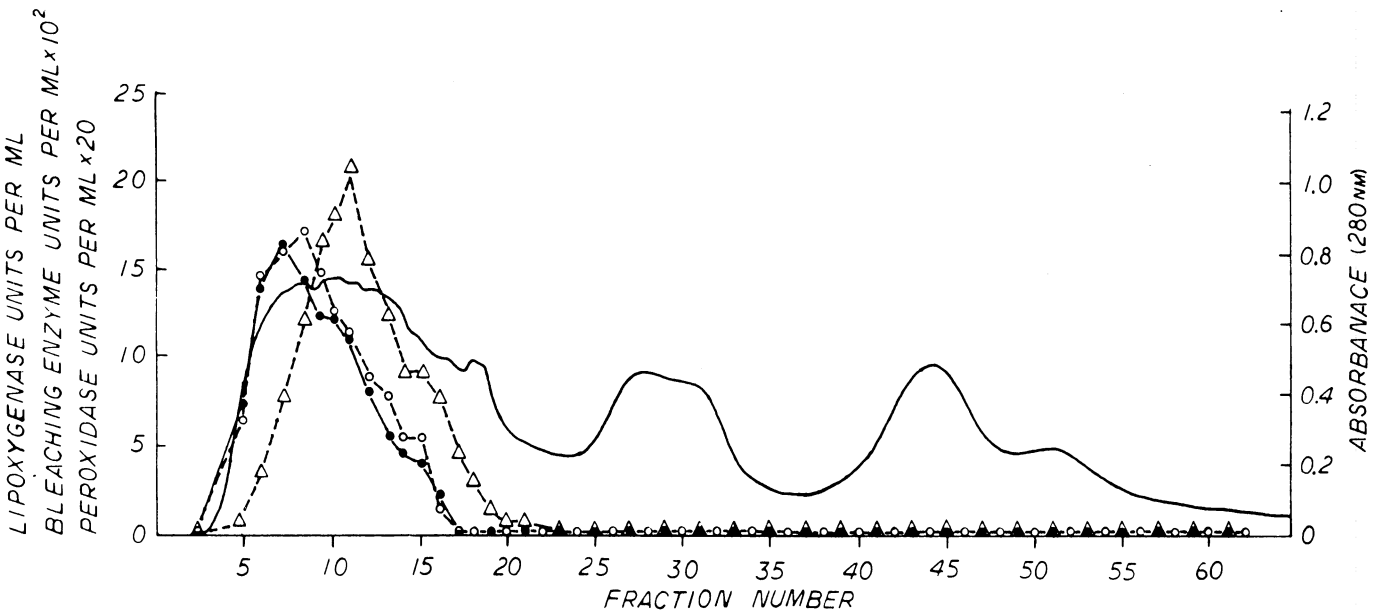

Fig. 2. DEAE-cellulose chromatography of crude durum flour lipoxygenase. – = Protein (A280); △ = lipoxygenase activity at pH 4.8; • = carotene bleaching activity at pH 9.2; o = peroxidase activity at pH 7.0.

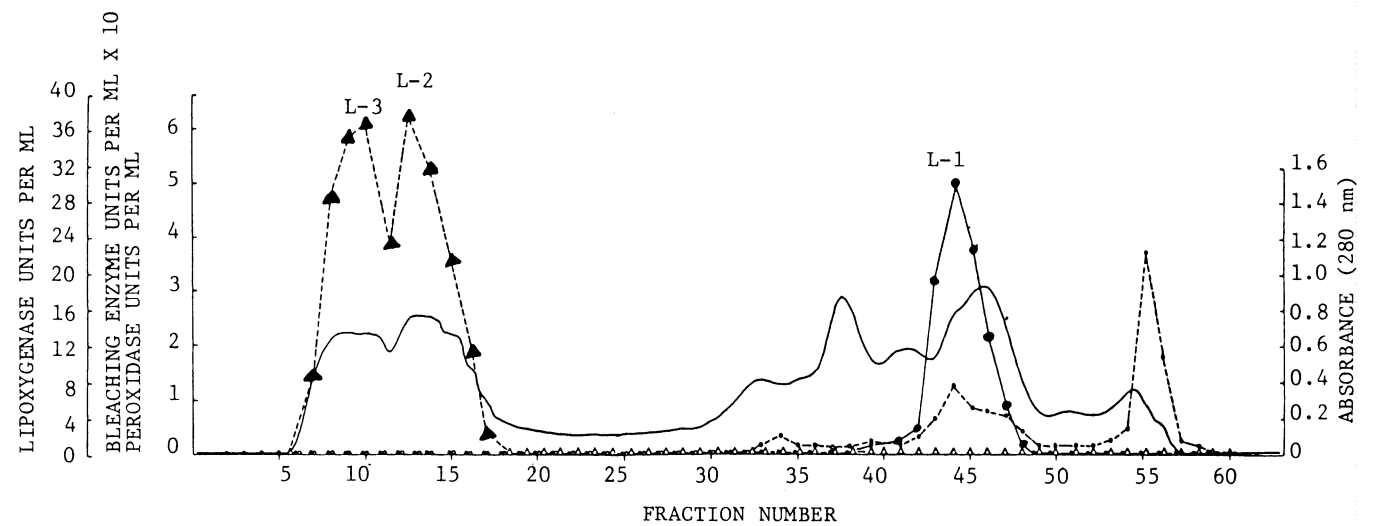

Fig. 3. Elution pattern of lipoxygenase isoenzymes from a CM-cellulose column. – = Protein (A280); ▲ = lipoxygenase activity at pH 4.8; • = carotene bleaching activity at pH 9.2.

On the basis of crude extract, a low degree of purification was attained, but on the basis of endosperm flour, the purification would be about five times higher. Low purification has also been reported in peas (Eriksson and Svensson 1970, Yoon and Klein 1979, Truong and Mendoza 1982), soybeans (Axelrod et al 1981), and red pepper fruits (Kanner et al 1977).

The relative lipoxygenase activity of the isoenzymes in crude extract from flour of Golden Ball durum wheat endosperm is calculated to be 21.3, 33.8, and 44.9%, respectively, for L-1, L-2, and L-3.

Analytical Electrophoresis of L-2 and L-3

The low rate of migration at acidic pH for both L-2 and L-3 (Fig. 5) indicates that both isoenzymes had low net positive charge at acidic pH, which is consistent with the elution pattern from CM-cellulose column (Fig. 3). Each isoenzyme separated into one major and one minor protein band at acidic pH, whereas one extra minor band was exhibited by both isoenzymes at a basic pH. Wallace and Wheeler (1979) reported that wheat germ lipoxygenase-1 dissociates into two active fractions when held at a pH above 7.0, with the amount of dissociated fraction being dependent apparently on the length of exposure. No attempt was made to isolate dissociated fraction from our L-2 and L-3, although extra bands were observed on electrophoresis at basic pH.

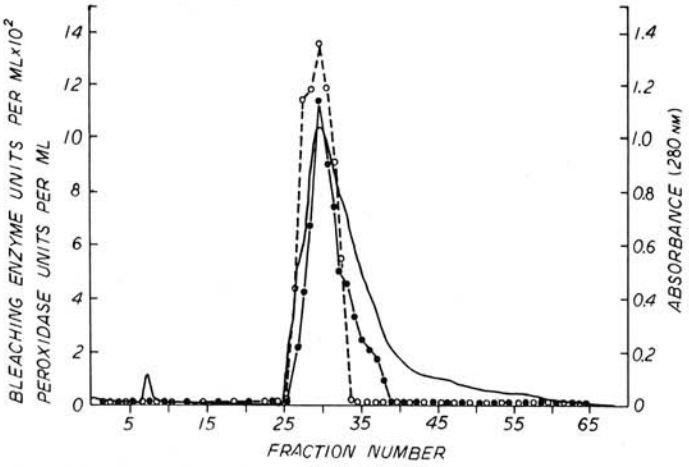

Fig. 4. Preparative disk polyacrylamide gel electrophoresis of the carotene bleaching fraction (L-1) obtained from CM-cellulose column. – = Protein (A280); • = carotene bleaching activity at pH 9.2; o = peroxidase activity at pH 7.0.

Carbohydrate Affinity Chromatography of L-1

Peroxidases from a variety of sources are glycoprotein with 18% carbohydrates reported in horseradish peroxidase (Shannon et al 1966) and differences in carbohydrate content reported in basic, neutral, and acidic isoenzymes of Japanese radish peroxidase

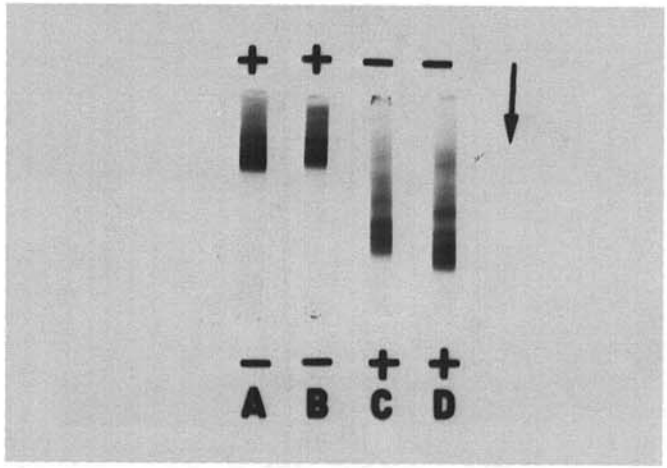

Fig. 5. Disk polyacrylamide gel electrophoresis of lipoxygenase. A = L-2, B = L-3, both at acidic pH; C = L-2, D = L-3, both at basic pH.

TABLE I
Summary of Purification of Lipoxygenase-1[a]

Procedure	Volume (ml)	Activity × 10^2 (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity × 10^3 (units/mg)	Yield (%)	Degree of Purification
Crude extract	9,287.0	0.8	74.3	3.1	2.6	100.0	1
$(NH_4)_2SO_4$ 0.3–0.6 saturation	216.0	17.0	36.7	27.0	6.3	49.4	2
DEAE-cellulose chromatography	38.1	152.0	57.9	73.0	20.8	77.9	8
CM-cellulose chromatography	6.3	282.0	17.8	62.6	45.0	24.0	17
Preparative disk electrophoresis	70.0	11.3	7.9	1.9	59.5	10.6	23

[a] Carotene bleaching activity.

TABLE II
Summary of Purification of Lipoxygenase-2 and -3

Procedure	Volume (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Specific Activity (units/mg)	Yield (%)	Purification (fold × 10)
Crude extract	9,287.0	0.6	5,572.2	3.1	1.9	100	1
$(NH_4)_2SO_4$ 0.3–0.6 saturation	216.0	27.5	5,940.0	27.0	10.2	106.6	5
DEAE-cellulose chromatography	38.1	101.7	3,874.8	73.0	13.9	69.5	7
CM-cellulose chromatography							
L-2	9.8	112.7	1,104.5	38.0	29.7	19.8	16
L-3	12.0	123.1	1,477.2	23.3	52.8	26.5	28

(Morita et al 1971). An affinity column containing concanavalin-A (Con A), a plant lectin, was used by Kahn et al (1981) to absorb and purify soluble potato peroxidase isoenzymes. In an attempt to separate the carotene bleaching from the peroxidase activity in L-1, a Con A-agarose affinity column was used (Fig. 6). From L-1 isoenzyme, two distinct protein peaks (A and C) and one tailing peak (B) were observed. All three peaks possessed carotene bleaching and pH 4.8 peroxidase activity. Peroxidase activity, however, was barely detectable until the need to activate the enzyme with $5 \times 10^{-4} M$ Ca^{2+} ions was discovered. This explains the continual loss of peroxidase activity during the purification. The presence of both enzyme activities in relatively similar amounts in peaks A, B, and C indicate that the peroxidase and carotene bleaching activity may reside on one protein molecule. The isoenzymes in the three peaks are designated as L-1A, L-1B, and L-1C.

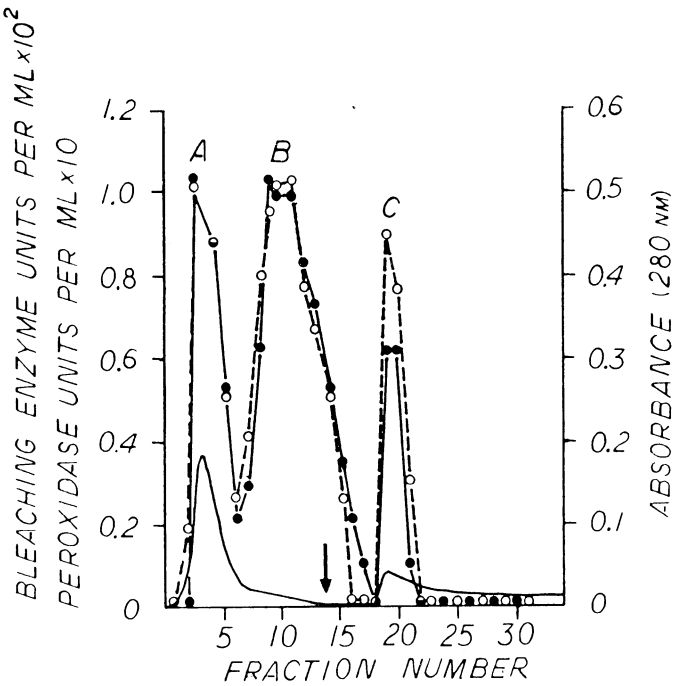

Fig. 6. Chromatography on glycosylex-A affinity column of the carotene bleaching factor (L-1) obtained from preparative disk polyacrylamide gel electrophoresis. – = Protein (A280); • = carotene bleaching activity at pH 9.2; o = peroxidase activity at pH 4.8.

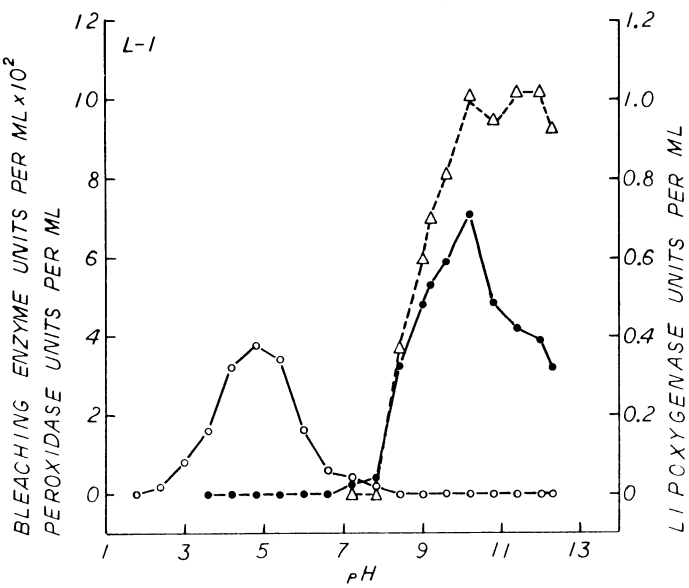

Fig. 7. Activity of L-1 lipoxygenase as a function of pH. △ = Lipoxygenase activity, reaction mixture contained linoleic acid ($2 \times 10^{-3} M$) and Tween 80 (0.48 μl/ml); • = β-carotene bleaching activity, reaction mixture contained β-carotene ($4.2 \times 10^{-6} M$), linoleic acid ($2 \times 10^{-3} M$) and Tween 80 (0.48 μl/ml); o = peroxidase activity, Ca^{2+} ($5 \times 10^{-4} M$) added.

pH Activity Profiles of L-1 and Affinity Column Fractions

The purified carotene bleaching factor (L-1) and its fractions from Glycosylex-A affinity chromatography were characterized by their pH profiles. The L-1 enzyme had very low lipoxygenase activity at the linoleic acid concentration normally used ($6.7 \times 10^{-5} M$) but was active when the linoleic acid concentration was raised to the level used for carotene bleaching ($2 \times 10^{-3} M$). The pH activity profiles of L-1 (Fig. 7) have an optimum at pH 10.2 and at 11.4 for lipoxygenase activity, an optimum at pH 10.2 for carotene bleaching activity, and one optimum for peroxidase activity at pH 4.8. The linoleic acid emulsion for bleaching assay that contains Tween 80 was also used for the lipoxygenase assay. The pH optima of L-1A, L-1B, and L-1C affinity column fractions of Fig. 6 were all similar to that of L-1. McDonald (1979) reported an optimum at pH 9.0 for lutein bleaching activity in durum semolina, but no corresponding lipoxygenase activity was found at the same pH region when using a much lower linoleate concentration than used here. In soybean, a large number of investigators have attributed an optimum at pH 9.0 to the Theorell enzyme or to soybean lipoxygenase-1.

The optimum activity at pH 4.8 for peroxidase (Fig. 7) corresponded with that of pH 5.0 found in wheat seeds (*T. aestivum* L.) and other cereals with 1,2-phenylenediamine as hydrogen donor (Fretzdorff 1980). Values ranging from pH 4 to 7 were reported for other plant peroxidase (Vamos-Vigyazo 1981).

pH Activity Profiles of L-2 and L-3

In determining the pH profiles of L-2 and L-3, the lipoxygenase activity was assayed at the normally low linoleic acid concentration

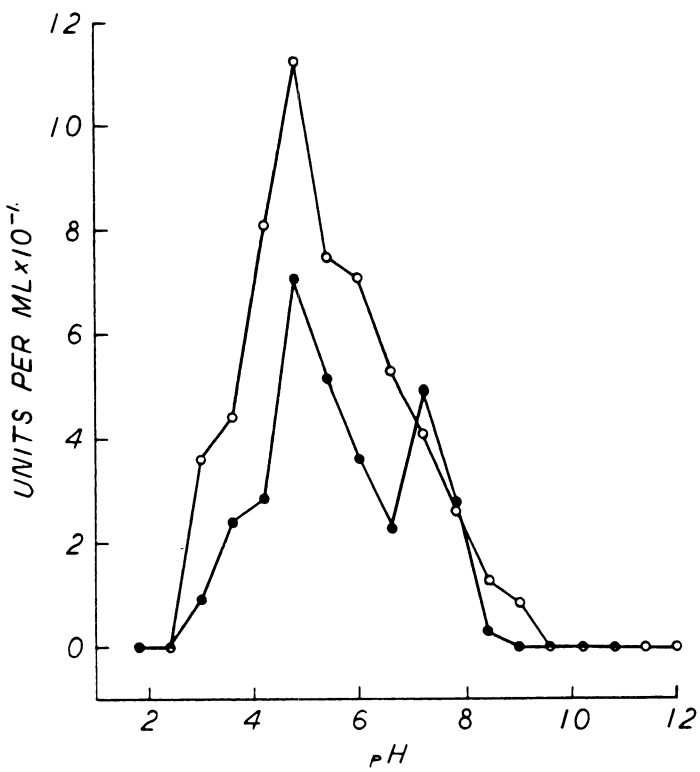

Fig. 8. Lipoxygenase activity of L-2 as a function of pH. o = Assay with $6.7 \times 10^{-5} M$ linoleic acid and Tween 20 (0.02 μl/ml); • = assayed with $2 \times 10^{-3} M$ linoleic acid and Tween 20 (0.48 μl/ml).

(6.7×10^{-5}), and at the higher concentration used for carotene bleaching ($2 \times 10^{-3} M$). The optimum for both L-2 and L-3 at low substrate concentration was pH 4.8, whereas, with high substrate concentration both had a second smaller activity peak at pH 7.2 (Figs. 8 and 9). The lipoxygenase activity of L-2 and L-3 at pH 4.8 with high concentrations of linoleic acid was 68 and 63%, respectively, of the activity with low substrate concentration. A similar observation of apparent substrate inhibition was also noted by Wallace and Wheeler (1975) for wheat germ lipoxygenase. The activity at pH 7.2 for L-2 and L-3 at high substrate concentration was respectively about 70 and 66% of the activity at pH 4.8.

In durum semolina, with $2.0 \times 10^{-2} M$ linoleic acid dispersed in Triton X-100 (2.1 μl/ml), Irvine and Anderson (1953a) reported an activity optimum at about pH 6.5 for lipoxygenase, using a manometric assay (Warburg Apparatus). Walsh et al (1970) reported a pH optimum at pH 5.9 and McDonald (1979) pH 4.8 with a diene conjugation assay and linoleic acid dispersed in Tween 20 at concentrations of 2.2×10^{-4} and $6.3 \times 10^{-5} M$, respectively. Also with the same assay method, Lulai and Baker (1976) reported an optimum at pH 6.0 for crude barley lipoxygenase with 5.5×10^{-5} M linoleic acid dispersed in Tween 20. For wheat germ isoenzymes, pH optima at $5.5 \times 10^{-5} M$ and $5 \times 10^{-3} M$ linoleic acid concentration have been reported, respectively, to be between pH 6–7 and pH 6–6.5 when polarographic types of assays are used (Wallace and Wheeler 1979, Nicolas et al 1982). Wallace and Wheeler (1975) also found that the pH optimum usually shifted to higher pH values with an increase in substrate concentration from 2.3×10^{-4} to $3.6 \times 10^{-3} M$, and they attributed this phenomenon to a pH-dependent inhibition by excess substrate. Ben Aziz et al (1970) also noted a shift in the activity optimum of soybean lipoxygenase at pH 7.5 and 9.0, with a threefold increase in substrate concentration.

In initial work, low β-carotene bleaching activity was observed for the isoenzymes L-2 and L-3. This low activity was found to be caused by inhibition by Tween 80 that was used to solubilize the substrates. The use of Tween 20 in place of Tween 80 overcame this problem. McDonald (1979) also reported low lutein bleaching activity for durum semolina at pH 3–7 when using Tween 80.

For carotene bleaching, the optimum activity of both L-2 and L-3 occurred at pH 6.6, with a smaller peak of activity at pH 4.8 (Fig. 10). Blain and Todd (1955), with rather unstable emulsions of carotene and linoleic acid (formed without emulsifiers), reported an optimum between pH 5.0 and 6.0 for carotene bleaching activity in crude extracts of wheat endosperm. They also noted that the concentration of linoleic acid required to obtain a comparable level of carotene destruction was greater than that for soybean extracts. With substrate dispersed by Tween 80, McDonald (1979) reported that two smaller peaks of lutein bleaching activity occurred at pH 6.0 and 4.0, with crude extract of durum semolina as an enzyme source. The optimum found here for carotene bleaching does not coincide exactly with that for lipoxygenase activity (Figs. 8 and 9), even when the linoleic acid concentrations are identical. This might indicate a difference in linoleic acid micelles in the two methods of assay or that bleaching is not caused by a simple random oxidation of β-carotene by linoleic acid-free radicals formed during the reaction, but that the β-carotene itself is being adsorbed in some way onto the enzyme molecule by ionizable groups of the enzyme or the conformation of the enzyme molecule is being changed by pH to a form that might cause stronger adsorption of the β-carotene. The active center for linoleic acid oxidation may be active in adsorbing the β-carotene, or another site close to it may be involved in adsorbing the β-carotene.

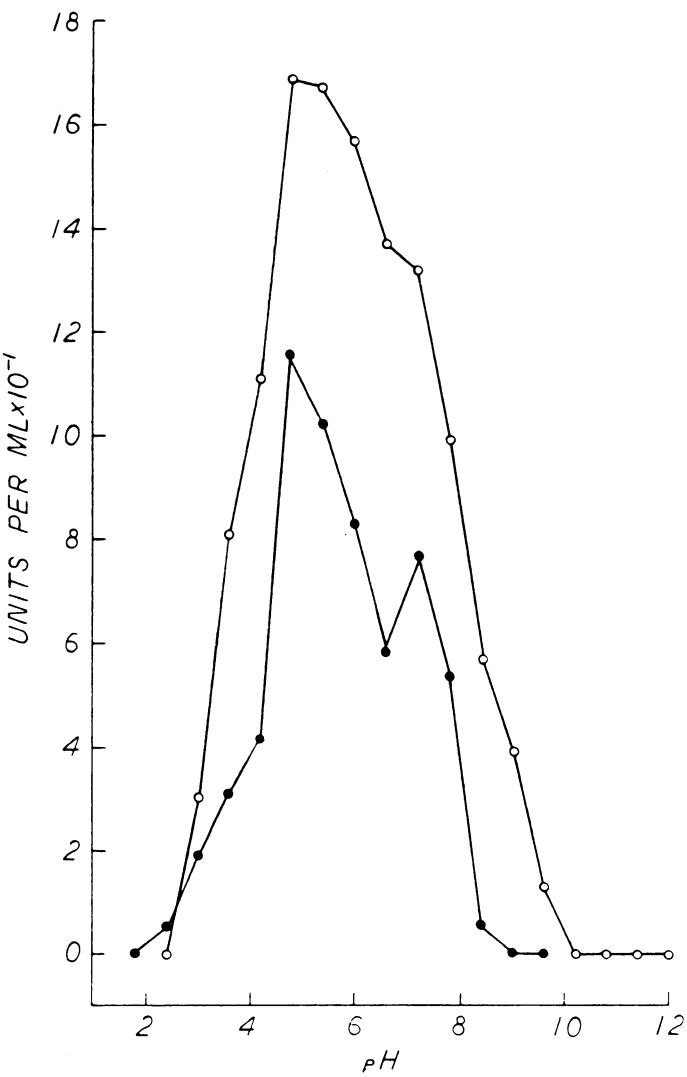

Fig. 9. Lipoxygenase activity of L-3 as a function of pH. o = Assay with $6.7 \times 10^{-5} M$ linoleic acid and Tween 20 (0.02 μl/ml); • = assayed with $2 \times 10^{-3} M$ linoleic acid and Tween 20 (0.48 μl/ml).

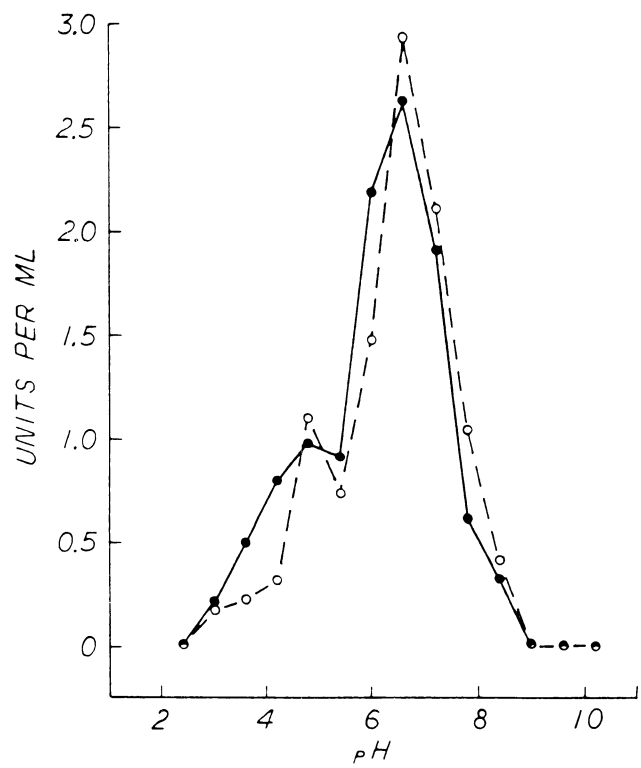

Fig. 10. Carotene bleaching activity as a function of pH. • = L-2; o = L-3, both reaction mixtures contained $4.2 \times 10^{-6} M$ β-carotene, $2.0 \times 10^{-3} M$ linoleic acid and Tween 20 (0.48 μl/ml).

SUMMARY AND CONCLUSIONS

Three fractions with lipoxygenase and carotene bleaching activities were isolated from endosperm flour of durum wheat. They have been designated as L-1, L-2, and L-3 isoenzymes.

The activity optima for L-1 are very high in pH, with lipoxygenase ones being pH 10.2 and 11.4, and the bleaching one being pH 10.2. This casts some doubt on the L-1 fraction being a classical lipoxygenase. The L-1 also has peroxidase activity with an activity optimum at pH 4.8. This peroxidase either must be part of the L-1 molecule or must be very closely associated, since it could not be separated by carbohydrate affinity chromatography. At the pH found during pasta processing, the peroxidase activity of L-1 should be active, whereas the lipoxygenase and carotene bleaching activities should be inactive.

The L-2 and L-3 isoenzymes have an activity optimum of pH 4.8 for lipoxygenase activity and pH 6.6 for carotene bleaching activity. These two isoenzymes should be primarily responsible for the loss of carotenoid pigments during pasta processing, since they should be active at the pH found in pasta dough. In the endosperm flour of Golden Ball durum, they apparently occur in considerably greater amounts than the L-1.

Work on the characterization of the three isoenzymes will be published in a later paper.

LITERATURE CITED

AMERICAN ASSOCIATION OF CEREAL CHEMISTS. 1983. Approved Methods of the AACC. Method 44-11, approved October 1976, reviewed October 1982; Method 44-15A, approved October 1975, revised October 1981; and Method 46-10, approved April 1961, revised October 1976, reviewed October 1982. The Association, St. Paul, MN.

AXELROD, B. 1974. Lipoxygenases. Adv. Chem. Ser. 136:324.

AXELROD, B., CHEESBROUGH, T. M., and LAAKSO, J. 1981. Lipoxygenase from soybeans. Page 441 in: Methods in Enzymology, Vol. 71. J. M. Lowenstein, ed. Academic Press, New York.

BEN AZIZ, A., GROSSMAN, S., ASCARELLI, I., and BUDOWSKI, P. 1970. Linoleate oxidation induced by lipoxygenase and heme proteins: A direct spectrophotometric assay. Anal. Biochem. 34:88.

BEN AZIZ, A., GROSSMAN, S., ASCARELLI, I., and BUDOWSKI, P. 1971. Carotene-bleaching activities of lipoxygenase and heme proteins as studied by a direct spectrophotometric method. Phytochemistry 10:1445.

BLAIN, J. A., and TODD, J. P. 1955. The lipoxygenase activity of wheat. J. Sci. Food Agric. 6:471.

CHANCE, B. 1954. The assay of catalases and peroxidases. Part II: Special methods. Page 407 in: Methods of Biochemical Analysis, Vol. 1. D. Glick, ed. Interscience, New York.

CHANCE, B., and MAEHLY, A. C. 1955. Assay of catalases and peroxidase. Page 764 in: Methods in Enzymology. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York.

COAKLEY, W. T., and JAMES, C. T. 1978. A simple linear transformation for the Folin-Lowry protein calibration curve to 1.0 mg/ml. Anal. Biochem. 85:90.

DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. N.Y. Acad. Sci. Ann. 121:404.

ERIKSSON, C. E., and SVENSSON, S. G. 1970. Lipoxygenase from peas. Purification and properties of the enzyme. Biochem. Biophys. Acta 198:449.

FRETZDORFF, B. 1980. Bestimmung der peroxidase Aktivitat in Getreide. Z. Lebensm. Unters. Forsch. 170:187.

IRVINE, G. N. 1959. Effect of temperature on the kinetics of the lipoxidase system of wheat. Cereal Chem. 36:146.

IRVINE, G. N. 1971. Durum wheat and pasta products. Page 777 in: Wheat Chemistry and Technology. Y. Pomeranz, ed. Am. Assoc. Cereal Chem., St. Paul, MN.

IRVINE, G. N., and ANDERSON, J. A. 1953a. Kinetic studies of the lipoxidase system of wheat. Cereal Chem. 30:247.

IRVINE, G. N., and ANDERSON, J. A. 1953b. Note on the lipoxidase activity of various North American wheats. Cereal Chem. 30:255.

IRVINE, G. N., and WINKLER, C. A. 1950. Factors affecting the color of macaroni. II. Kinetic studies of pigment destruction during mixing. Cereal Chem. 27:209.

KAHN, V., GOLDSHMIDT, S., AMIR, J., and GRANIT, R. 1981. Some biochemical properties of soluble and bound potato tuber peroxidase. J. Food Sci. 46:756.

KANNER, J. K., MENDEL, H., and BUDOWSKI, P. 1977. Carotene oxidizing factors in the red pepper fruits (*Capsicum annuum* L.): Peroxidase activity. J. Food Sci. 42:1549.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

LULAI, E. C., and BAKER, C. W. 1976. Physicochemical characterization of barley lipoxygenase. Cereal Chem. 53:777.

MAEHLY, A. C., and CHANCE, B. 1954. Catalases and peroxidases. Part I: General assay methods. Page 357 in: Methods of Biochemical Analysis, Vol. 1. D. Glick, ed. Interscience, New York.

McDONALD, C. E. 1979. Lipoxygenase and lutein bleaching activity of durum wheat semolina. Cereal Chem. 56:84.

MORITA, Y., YOSHIDA, C., and MAEDA, Y. 1971. Properties and structure of peroxidase isoenzymes of Japanese radish. Agric. Biol. Chem. 35:1074.

NICOLAS, J., AUTRAN, M., and DRAPRON, R. 1982. Purification and some properties of wheat germ lipoxygenase. J. Sci. Food Agric. 33:365.

PRIVETT, O. S., CHRISTENSE, N., and LUNDBERG, W. O. 1955. Products of the lipoxidase-catalyzed oxidation of sodium linoleate. J. Am. Oil Chem. Soc. 32:505.

REISFELD, R. A., LEWIS, U. T., and WILLIAMS, D. E. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281.

SHANNON, L. M., KAY, E., and LEW, J. W. 1966. Peroxidase isoenzymes from horseradish roots. I. Isolation and physical properties. J. Biol. Chem. 241:2166.

SHARON, N., and LIS, H. 1972. Lectins: Cell-agglutinating and sugar specific proteins. Science 177:949.

SURREY, K. 1964. Spectrophotometric method for determination of lipoxidase activity. Plant Physiol. 39:65.

TRUONG, V. D., and MENDOZA, E. M. Y. 1982. Purification and characterization of two lipoxygenase isoenzymes from cowpea. J. Agric. Food Chem. 30:54.

VAMOS-VIGYAZO, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC Crit. Rev. Food Sci. Nutr. 15:49.

WALLACE, J. M., and WHEELER, E. L. 1975. Lipoxygenase from wheat. An examination of its reaction characteristics. J. Agric. Food Chem. 23:146.

WALLACE, J. M., and WHEELER, E. L. 1979. Two lipoxygenase isoenzymes and an activator in wheat germ. Phytochemistry 18:389.

WALSH, D. E., YOUNGS, V. L., and GILLES, K. A. 1970. Inhibition of durum wheat lipoxidase with L-ascorbic acid. Cereal Chem. 47:119.

WEBER, F., LASKAWY, G., and GROSCH, W. 1973. Enzymatischer Carotinabbau in Erbsen, Sojabohnen, Weizen and Leinsumen. Z. Lebensm. Unters. Forsch. 152:324.

YOON, S., and KLEIN, B. P. 1979. Some properties of pea lipoxygenase isoenzymes. J. Agric. Food Chem. 37:955.

ZIMMERMAN, D. C., and VICK, B. A. 1970. Hydroperoxide isomerase. A new enzyme in lipid metabolism. Plant Physiol. 46:445.

[Received July 15, 1983. Accepted March 30, 1984]