Purification and Characterization of a Lipoxygenase Enzyme from Durum Wheat Semolina

Roberto Barone,[†] Raffaella Briante,[†] Sabato D'Auria,[†] Ferdinando Febbraio,[†] Carlo Vaccaro,[†] Luigi Del Giudice,[‡] Grazia Maria Borrelli,[§] Natale Di Fonzo,[§] and Roberto Nucci*[,†]

Istituto di Biochimica delle Proteine ed Enzimologia, Consiglio Nazionale delle Ricerche, via Marconi 10, 80125 Napoli, Italy, Istituto Internazionale di Genetica e Biofisica, Consiglio Nazionale delle Ricerche, via Marconi 12, 80125 Napoli, Italy, and Istituto Sperimentale per la Cerealicoltura, Sezione di Foggia, Italy

Purification of a lipoxygenase enzyme from the cultivar Tresor of durum wheat semolina (*Triticum turgidum* var. durum Desf) was reinvestigated furnishing a new procedure. The 895-fold purified homogeneous enzyme showed a monomeric structure with a molecular mass of 95 ± 5 kDa. Among the substrates tested, linoleic acid showed the highest k_{cat}/K_m value; a β-carotene bleaching activity was also detected. The enzyme optimal activity was at pH 6.8 on linoleic acid as substrate and at pH 5.2 for the bleaching activity on β-carotene, both assayed at 25 °C. The dependence of lipoxygenase activity on temperature showed a maximum at 40 °C for linoleic acid and at 60 °C for bleaching activity on β-carotene. The amino acid composition showed the presence of only one tryptophan residue per monomer. Far-UV circular dichroism studies carried out at 25 °C in acidic, neutral, and basic regions revealed that the protein possesses a secondary structure content with a high percentage of α- and β-structures. Near-UV circular dichroism, at 25 °C and at the same pH values, pointed out a strong perturbation of the tertiary structure in the acidic and basic regions compared to the neutral pH condition. Moreover, far-UV CD spectra studying the effects of the temperature on α-helix content revealed that the melting point of the α-helix is at 60 °C at pH 5.0, whereas it was at 50 °C at pH 6.8 and 9.0. The NH_2-terminal sequence allowed a homology comparison with other lipoxygenase sequences from mammalian and vegetable sources.

Keywords: *Lipoxygenase; durum wheat; kinetics; circular dichroism; sequence homology*

INTRODUCTION

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a group of enzymes that catalyze the regio- and stereoselective dioxygenation of polyunsaturated fatty acids with a (*Z*,*Z*)-1,4-pentadiene system: the resulting products are optically active, conjugated (*Z*,*E*)-diene hydroperoxy derivatives (Gaffney, 1996). Lipoxygenases are found both in plants and in animals (Hildebrand, 1989); their occurrence in plants has been implicated in flavor and odor formation, in fruit ripening, in abscission, and in senescence. They may also play a role in responses to pest attack and wounding (Hildebrand, 1989; Gardner, 1991; Siedow, 1991). Some lipoxygenase isoenzymes may also function as vegetative storage proteins (Kato et al., 1993). The involvement of lipoxygenase in determining the quality of wheat product, particularly with regard to the yellow pigment content, which is lost during processing of the milled durum product, semolina, into the pasta product, has been widely studied previously (Irvine, 1971; McDonald, 1979; Talia and Sagi, 1987). A bright yellow color of pasta is in fact due to lutein, a lipid carotenoid pigment influencing the quality of durum wheat grain for pasta production (Irvine, 1971). The color of pasta is a function of both semolina color and loss of pigment during processing (McDonald, 1979). Loss of yellow lutein was supposed to be due to enzymatic bleaching of carotene by coupled oxidation with unsaturated fats due to lipoxygenase activity (Irvine, 1971; McDonald, 1979). Lipoxygenase occurrence in higher plants used for food is of special interest because these enzymes convert polyunsaturated fatty acids into hydroperoxides; the latter may be decomposed to form products with characteristic, both desirable and undesirable, tastes and smells, such as *trans*-2-nonenal, in part responsible for the development of the stale flavor in beer during storage (Tressl et al., 1978). In animals, lipid hydroperoxides and products of their conversion derived through the lipoxygenase pathway are involved in many physiological and pathological processes. They form precursors of important classes of chemical messengers, such as leukotrienes and lipoxins (Anderson, 1989).

Lipoxygenases have been isolated from many plants but have been most studied in soybean seeds, in which they constitute 1−2% of the seed protein in three isoenzymatic forms (Lox-1, Lox-2, and Lox-3) which were distinguished by pH optimum, substrate specificity, product formation, and stability (Axelrod et al., 1981; Shibata et al., 1987, 1988; Yenofsky et al., 1988). Also in wheat, three lipoxygenase isoenzymes, L-1, L-2, and L-3, have been isolated and studied at the biochemical level. However, the method described for the purification of durum wheat lipoxygenases resulted in a very

* Author to whom correspondence should be addressed (telephone +39/081/7257300; fax +39/081/7257300; e-mail nucci@dafne.ibpe.na.cnr.it).

[†] Istituto di Biochimica delle Proteine ed Enzimologia.

[‡] Istituto Internazionale di Genetica e Biofisica.

[§] Istituto Sperimentale per la Cerealicoltura.

10.1021/jf980853z CCC: $18.00
Published on Web 04/17/1999

long procedure with impure enzyme extracts (Hsieh and McDonald, 1984).

We describe here a new method for purifying a lipoxygenase activity by a simple procedure from the cultivar Tresor of durum wheat plant species (Lox). The present data originating from the biochemical characterization showed that the Lox activity purified by us appeared to be different from the three isoenzymes previously extracted from durum wheat (Hsieh and McDonald, 1984).

MATERIALS AND METHODS

Plant Material. The cultivar Tresor of durum wheat (*Triticum turgidum* var. durum Desf) grown in 1996 in Faeto (Foggia, Italy) was used throughout this study because it presented a high level of Lox activity from seed crude extract (Manna et al., 1998).

Semolina Production. For the evaluation of LOX activity, the seeds were tempered to 17% moisture and milled on a BONA experimental mill with 42 and 54 GG sieves, with four rolls to give 50−60% yield of semolina.

Chemicals. All chemicals were of reagent grade. DEAE Sepharose F.F., Superdex G75, and C-4 Vydac reverse phase for HPLC were purchased from Pharmacia Biotechnology (Uppsala, Sweden); α-linoleic and α-linolenic acid, α-arachidonic acid, methyl linoleate, and β-carotene were from Sigma (St. Louis, MO). Bovine serum albumin and molecular mass standards were from Boehringer-Mannheim (Mannheim, Germany). The low molecular weight electrophoresis calibration kit was from Pharmacia Biotechnology-Uppsala (Sweden). The ultrafiltration apparatus and YM-30 membrane were from Amicon Corp. (Danvers, MA).

Enzyme Assay. Lox activity was assayed spectrophotometrically at 25 °C by measuring the increase in absorbance at 234 nm for linoleic and linolenic acids and at 238 nm for arachidonic acid, in a 1.0 mL quartz cuvette (Helma, Jamaica, NY) by a Cary E1 Varian spectrophotometer equipped for controlling temperature by Peltier effect (Varian, VIC, Australia). The reaction mixture contained 50 mM sodium phosphate (pH 6.8) and 0.1 μg of enzyme/mL of final volume. The final concentration of the substrate, linoleic acid, was 92.7 μM according to Vick et al. (1987) as well as for the linolenic and arachidonic acids, respectively. For each substrate used, we have prepared a mother solution containing 9.27 mM, 2.5 μL/mL Tween 20, which was stored at 4 °C under nitrogen. One unit of activity is defined as the amount of enzyme that originates 1 μmol of hydroperoxide formed per minute at 25 °C. The ϵ_M for the linoleic acid is 2.5×10^4 M^{-1} cm^{-1} (Axelrod et al., 1981).

Carotene Bleaching Assay. A mother solution was prepared containing 0.993 mM β-carotene in 50 mM sodium phosphate (pH 6.5), 0.225% EDTA, 18 μL/mL Tween 80, and 0.25% $CHCl_3$ and was stored at −20 °C. The reaction mixture contained 50 mM sodium acetate buffer (pH 5.2), and the enzyme assay was performed at 25 °C by measuring the decrease in absorbance at 460 nm using 0.86 μg of enzyme/mL of final volume, 13.96 μM β-carotene, and 2.31 mM linoleic acid, according to the method of Ben Aziz et al. (1971) with the modifications indicated by Hsieh et al. (1984). One unit of activity was equivalent to the destruction of 1 μmol of β-carotene/min at 25 °C. The ϵ_M used for β-carotene is 1.2×10^5 M^{-1} cm^{-1}.

Protein Determination. Protein concentration was determined according to the assay of Folin−Ciocalteu (Lowry et al., 1951), using bovine serum albumin as standard and by the molar extinction coefficient of purified enzyme ($\epsilon_M = 1.3 \times 10^5$ M^{-1} cm^{-1}).

Purification of Lox Activity. *Preparation of Crude Extract*. An amount of 560 g of durum wheat semolina, variety Tresor, was suspended in 1.5 L of 20 mM sodium phosphate (pH 6.8) and broken by magnetic stirring. Cell debris was removed by centrifugation in a Sorvall centrifuge at 8000 rpm for 30 min.

Ammonium sulfate was added to the supernatant to a 30% saturation, and the mixture was stirred for 30 min at 4 °C. The solution was centrifuged by a Sorvall at 16300*g* for 10 min at 4 °C and the pellet discarded. Further addition of ammonium sulfate to a 60% saturation produced a mixture that was stirred for 30 min at 4 °C. After precipitation, the mixture was centrifuged by a Sorvall at 16300*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet, dissolved in 30 mL of 0.02 M sodium phosphate buffer (pH 6.8), represents the crude extract.

Column Chromatography. The protein solution was loaded, at 4 °C, on a DEAE-Sepharose F.F. column 26/60, equilibrated with 0.01 M sodium phosphate buffer (pH 6.8), using a flow rate of 3 mL/min. The enzyme, eluted by a linear gradient from 0.01 to 0.15 M sodium phosphate buffer (pH 6.8), was recovered at ∼40 mM sodium phosphate (pH 6.8). The active fractions were pooled and concentrated by precipitation at 60% ammonium sulfate. The precipitate has been dissolved in a buffer of 0.01 M sodium phosphate (pH 6.8) and dialyzed against 2 L of 0.01 M sodium phosphate (pH 6.8). The dialysate was concentrated by an ultrafiltration apparatus equipped with a YM-30 membrane to a final volume of 2.5 mL. The sample so obtained was loaded on a Superdex-G-75 column, 2.6 × 60 cm, equilibrated with 0.05 M sodium phosphate buffer (pH 6.8) and 0.15 M NaCl, using a flow rate of 0.3 mL/min. The active fractions were dialyzed against 2 L of 0.01 M sodium phosphate (pH 6.8) and concentrated with Centriplus 30. The enzymatic solution (1.21 mg/mL) was stored at 4 °C. The enzyme showed a final specific activity of 42.98 units/mg.

Determination of Protein Homogeneity by HPLC. About 10 μg of the purified enzyme was loaded on a reverse phase Vydac C-4 column equilibrated in H_2O + 0.1% trifluoroacetic acid and eluted by a linear gradient from 0 to 100% CH_3CN + 0.1% trifluoroacetic acid.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was used to determine Lox purity and its quaternary structure. A 10% (w/v) separation slab gel was used with Laemmli's continuous buffer system (Laemmli, 1970). Phosphorylase *b* (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactoalbumin (14.4 kDa) were used as standards. Proteins were stained with Coomassie Brilliant Blue R-250.

Molecular Mass Determination. To estimate the molecular mass of the durum wheat Lox, gel filtration was carried out according to the procedure described by Andrews (1964); 0.2 mL of the purified enzyme (10.0 mg/mL) was loaded on a Superdex G-200 column by a FPLC system, at a flow rate of 0.6 mL/min. The column was previously equilibrated with 50 mM sodium phosphate/0.15 mM NaCl (pH 6.8) and calibrated with the following standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Amino Acid Analysis. The amino acid composition has been determined on hydrolyzed samples by 6 N HCl at 110 °C for 20 h in sealed vials under vacuum. The sample has been lyophilized and suspended in 0.2 M sodium citrate buffer (pH 2.2) and injected in a Beckman automatic analyzer (Gold System) on a Pharmacia HPLC ionic exchange (Na^+) high-resolution column specific for amino acid analysis. The tryptophan residues have been determined on hydrolyzed samples by 3 N mercaptoethanolsulfonic acid (MESNA) at 110 °C for 20 h in sealed vials under vacuum. The hydrolysate has been titrated by dilution 1:1 with 2 M NaOH and lyophilized. The sample has been suspended in a final volume of 1 mL by 0.2 M sodium citrate and injected in a Beckman automatic analyzer (Gold System).

Effect of pH, Ionic Strength, and Temperature. The optimum pH for the Lox activity, using linoleic acid as substrate was determined at 25 °C in the pH range between 3.0 and 10.0, using the following buffers: glycine−NaOH, sodium acetate, sodium phosphate, sodium borate, and glycine−HCl, all at 50 mM final concentration. About 1.0 μg of the enzyme was used in a final assay volume of 1.0 mL. The

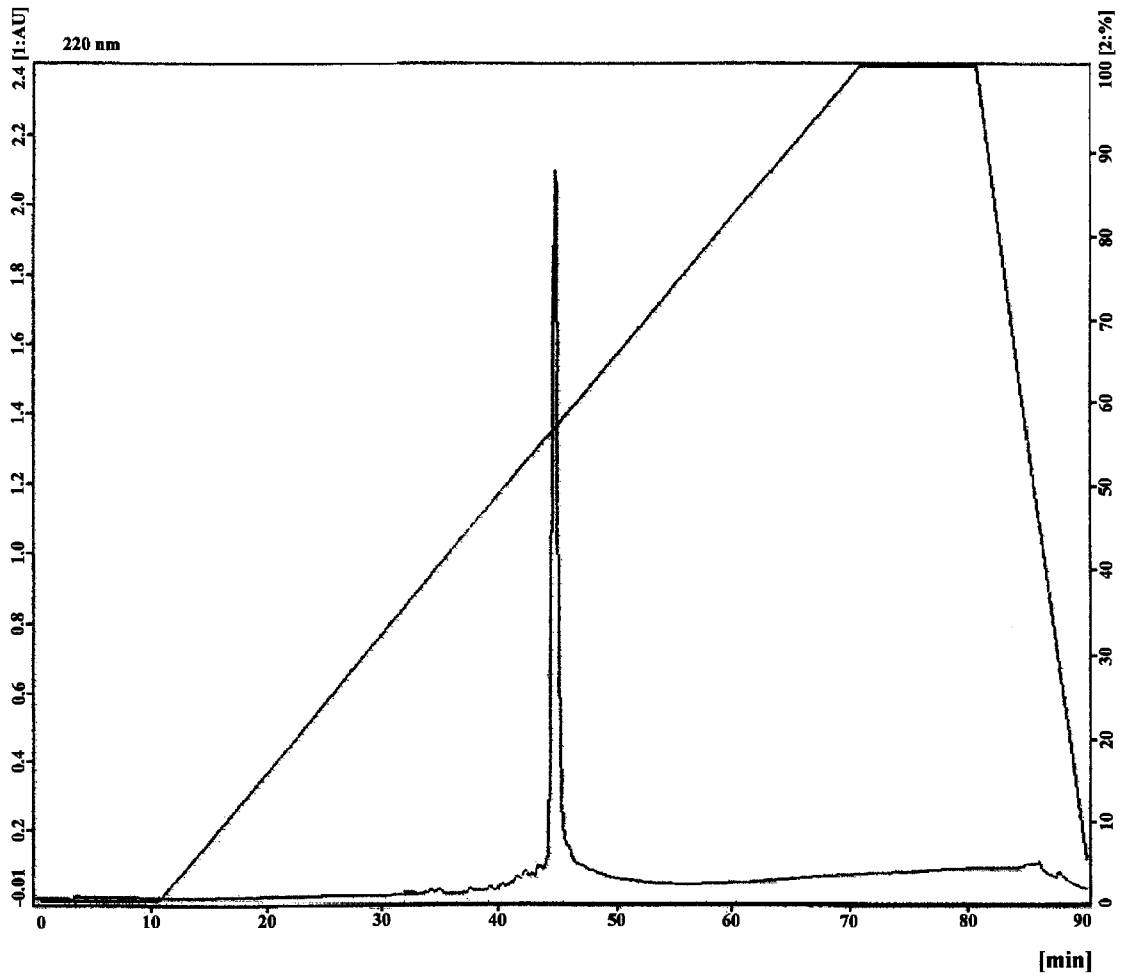

Figure 1. RP-HPLC of purified Lox (10 μg) by a linear gradient. For sample elution see Materials and Methods.

optimum pH for the β-carotene bleaching activity was determined at 25 °C using the above assay in the presence of the same mentioned buffers.

The effect of ionic strength on enzymatic activity, using linoleic acid as substrate, has been tested using different sodium phosphate (pH 6.8) concentrations from 5 to 100 mM in the standard assay mixture, in the presence of 1.0 μg of enzyme.

Enzyme thermophilicity was tested in the standard assay mixture from 5 to 80 °C at intervals of 5 °C, using 1.0 μg of enzyme and linoleic acid as substrate in plugged quartz cuvettes. The effects of temperature on β-carotene bleaching activity, using linoleic acid as substrate, have been studied in a temperature range from 5 to 90 °C every 10 °C, using 0.86 μg of enzyme in the standard assay mixture. The standard assay mixtures were preincubated for 5 min at the indicated temperatures in plugged quartz cuvettes before enzyme addition.

Determination of Kinetic Parameters. The kinetic constants of Lox activity were measured under the standard assay conditions, using substrate concentrations in the range 1−300 μM, for linoleic, linolenic, and arachidonic acid. The enzyme concentration was 0.1 μg/mL of reaction mixture in all cases. All determinations were done in duplicate, and the respective kinetic parameters were evaluated from Lineweaver−Burk plots. The kinetic results were analyzed with the Grafit program (Leatherbarrow, 1990). This program uses linear regression to calculate the best fit line through a series of data points.

Storage Stability. The stability of Lox activity has been followed for 6 days of incubation of the enzyme at 4 °C in absence and in the presence of 1 mM β-mercaptoethanol, 1 mM dithiothreitol, and 200 ppm of L-ascorbic acid using the standard assay in the presence of 1.0 μg of enzyme and at 25 °C for 4 days in absence of effectors in the standard assay.

Circular Dichroism. Measurements were performed on homogeneous samples at a protein concentration of 0.6 mg/mL in 5 mM sodium phosphate (pH 6.8) in the near-UV and of 0.1 mg/mL in the far-UV regions, respectively. The experiments at different temperatures in the range from 10 to 85 °C were carried on in plugged quartz cuvettes, incubating the samples for 5 min. A Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) equipped with temperature-controlled liquid system Neslab RTE-110 (Neslab Instruments, Portsmouth) calibrated with a standard solution of (+)-10-camphorsulfonic acid was used. Far-UV spectra from 240 to 200 nm were recorded using 0.1 cm light path quartz cuvettes (Helma), under nitrogen flow. Near-UV spectra from 250 to 320 nm were recorded using 1.0 cm light path quartz cuvettes (Helma). A spectral acquisition spacing of 0.1 nm (1.0 nm bandwidth) was used, and the photomultiplier absorbance did not exceed 600 V. Each spectrum was averaged 10 times and smoothed with System Software ver. 1.00 (Jasco).

NH_2-Terminal Sequence. A lyophilized protein sample, 300 nmol, was subjected to automatic Edman degradation by an Applied Biosystems 477 A protein sequencer, equipped on line with a model 120 A phenylthiohydantoin analyzer. Degradation proceeded for 15 cycles according to the manufacturer's instruction and produced identifiable amino acid residues.

RESULTS AND DISCUSSION

Enzyme Purification. The results of a typical purification procedure are summarized in Table 1. The Lox was purified 895-fold; the DEAE-Sepharose F.F.

Table 1. Durum Wheat Lipoxygenase Purification Procedure

purifn step	total protein (mg)	total act. (units)[a]	sp act. (units/mg)	yield (%)	purifn (-fold)
crude extract	32801	1574.0	0.0480	100	1.00
anionic exchange	321.3	1295.9	4.036	82.3	84.08
gel filtration	20.6	884.0	42.98	68.2	895.4

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

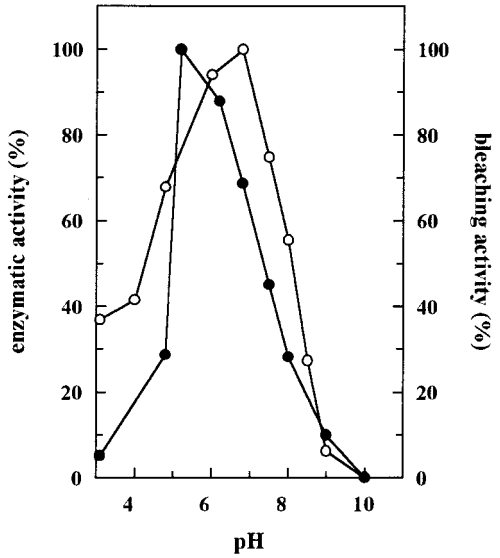

Figure 2. Dependence of Lox (○) and β-carotene bleaching (●) activities on pH. Standard assays, except for the buffer employed, were used. One hundred percent enzymatic activity corresponds to 0.043 unit/μg for Lox (○) and β-carotene bleaching (●) activities.

represents the crucial step in the purification with 82% yield. The final purification step was performed by Superdex G-75 with a final yield of 68% of enzyme activity. Lox purity was tested by a reverse phase HPLC column (Figure 1). For the homogeneous Lox activity from durum wheat we obtained a specific activity of 42.98 units/mg. The high purification after the ionic exchange chromatography was a result of a good interaction between the enzyme and this matrix. Preliminary evidence on the molecular masses of the proteins that migrate together with Lox activity band has justified the use of the gel filtration chromatography. Also in this case we got a good enzyme recovery after this step.

Molar Extinction Coefficient Determination. The molar extinction coefficient ϵ_M, calculated by using the Lambert−Beer law at a wavelength of 280 nm and at 25 °C, equals 1.3×10^5 M^{-1} cm^{-1}. The protein concentration is 16.7 μg/mL. This value is comparable to that found for LOX-1 from soybean (1.4×10^5 M^{-1} cm^{-1}) (Axelrod et al., 1981).

pH Profiles. Lox and β-carotene bleaching activities were assayed under standard conditions on linoleic acid, in a range of pH from 3.0 to 10.0. Lox activity showed its maximum at pH 6.8 in 0.05 M sodium phosphate buffer, but it is anyhow appreciable in the pH range from 4.8 and 7.5. This result is comparable with those described in the literature for lipoxygenases from barley (Lulai and Baker, 1976; van Aarle et al., 1991) and from wheat flour (Irvine and Anderson, 1953) and for isoenzymes L-2 and L-3 from soybean (Axelrod et al., 1981). The pH profile of this Lox activity is different from that

Table 2. Substrate Specificity of the Durum Wheat Lipoxygenase

substrate	K_m (μM)	k_{cat} (s^{-1})	V_{max} (μmol min^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
linoleic acid	11.25	156.26	0.09869	13.89
linolenic acid	120.28	7.01	0.00443	0.058
arachidonic acid	16.47	7.88	0.00497	0.478

Table 3. Amino Acid Composition of Lox

amino acid	nearest integer	amino acid	nearest integer
Asp/Asn	96	Met	22
Thr	44	Ile	22
Ser	89	Leu	89
Glu/Gln	100	Tyr	33
Pro	7	Phe	52
Gly	70	Lys	60
Ala	70	His	29
Cys[a]	nd	Trp[b]	1
Val	37	Arg	37

[a] The cysteine residues were not determined by the used method. [b] The tryptophan residues were determined by the MESNA method, as described in the text.

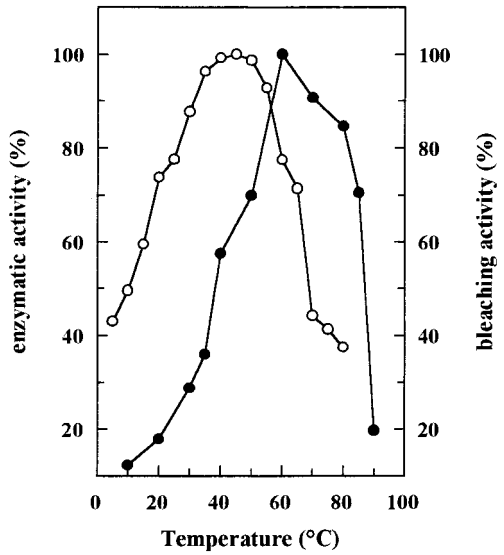

Figure 3. Dependence of Lox (○) and β-carotene bleaching (●) activities on temperature. One hundred percent enzymatic activity corresponds to 0.055 unit/μg for Lox (○) and 0.172 unit/μg for β-carotene bleaching (●) activities.

of the three isoenzymes present in durum wheat flour described by Hsieh (Hsieh and McDonald, 1984), probably due to the different extraction buffer and/or cultivar variety used. In contrast, for the β-carotene bleaching activity, the maximum was revealed at pH 5.2 in 0.05 M sodium acetate buffer (Figure 2)

Effect of Ionic Strength. In determining the effect of ionic strength, the Lox activity was assayed using linoleic acid as substrate in the presence of different concentrations of sodium phosphate (pH 6.8) at 25 °C. The enzymatic activity is present in all ranges of concentrations, showing a maximum at 50 mM of the above buffer with an increase of 30% in comparison to the activity at 5 mM (data not shown).

Kinetic Analysis and Substrate Specificity. A kinetic characterization of Lox activity was carried out in relation to three different substrates: linoleic acid (C 18:2), linolenic acid (C 18:3), and arachidonic acid (C 20:4), using 0.05 M sodium phosphate buffer (pH 6.8). The kinetic constants, K_m and k_{cat}, are indicated in Table 2. The K_m values for linoleic and arachidonic acids were

Lox			.....QSALLVLTVVLMIKNLF.....	
lipoxygenase	*- potato*	1	MIGQITSGLFGGHDD..SKKVKGTVVMMNKNVLDFT.....	34
lipoxygenase	*- common tobacco*	1	MFLEKIVDAITGKDD..GKKVKGTVVLMKKNVLDFT.....	34
lipoxygenase AtLox1	*- Arabidopsis thaliana*	1	MFGELRDLLTGGGNETTTKKVKGTVVLMKKNVLDFN.....	36
lipoxygenase	*- soybean*	1	.....MFGIFDK..GQKIKGTVVLMPKNVLDFNAITSIGKGGVIDTA	40
lipoxygenase	*- kidney bean (phavu)*	1	.....MFGILNR..GHKIKGTVVLMTKNVFDFNEFVSTTRGGIVGAA	40
lipoxygenase	*- garden pea*	1	....MFSGVTGILNR..GHKIKGTVVLMRKNVLDINSLTT.....V....	35
lipoxygenase 3	*- soybean*	1	.....MLGGLLHR..GHKIKGTVVLMRKNVLDVNSVTS.....V....	32
lipoxygenase 1	*- soybean*	1	.....MFSAGHKIKGTVVLMPKNELE.....	21
lipoxygenase 2	*- soybean*	1	..MFSVPGVSGILNRGGGHKIKGTVVLMRKNVLDFNSVADLTKGNV....	44
lipoxygenase pea2	*- garden pea*	1	....MFPNVTGLLNK..GHKIRGTVVLMRKNVLDFNTIVSIGGGNV....	40

Figure 4. Comparison among 17 residue motifs of the Lox NH_2-terminal sequence and other plant lipoxygenases. Bold letters refer to higly conserved amino acids.

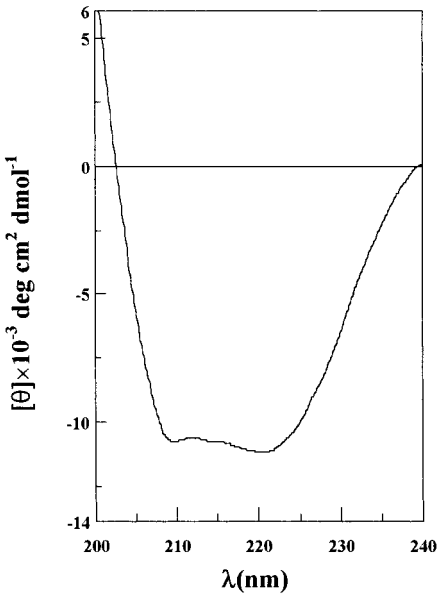

Figure 5. Far-UV CD spectrum of Lox at pH 6.8.

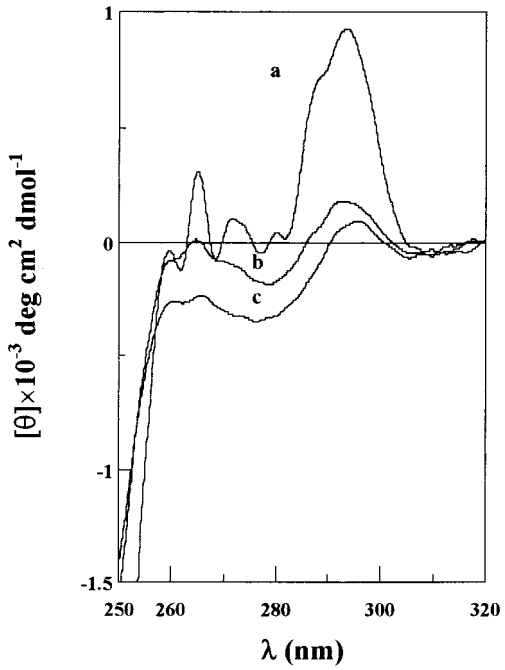

Figure 6. Near-UV CD spectra of Lox at three different pH values: (a) at pH 6.8; (b) at pH 5.0; (c) at pH 9.0.

11.25 and 16.47 μM, respectively, indicating high enzyme affinity for these substrates. Moreover, the analysis of k_{cat}/K_m ratios showed that linoleic acid was the preferred substrate; in contrast, linolenic acid was a poor substrate, showing a higher K_m value and a much lower k_{cat} value. Concerning the activity on arachidonic acid, despite the good affinity shown for this substrate, the low k_{cat} value points out that the catalysis is poor. No activity was observed in the assay using methyl linoleate as a substrate, as described first by Verhue et al. (1972) for a soybean lipoxygenase, suggesting that the presence on the above substrate of a negative charge due to the carboxylic group plays a relevant role in the interaction inside the catalytic site. The K_m value for linoleic acid is close to the values of 13 and 19 μM, respectively reported for Lox-1 and -2 from germinating barley (Yang et al., 1993); conversely, our enzyme showed a smaller affinity when assayed on linolenic acid.

Effect of Linoleic Acid on β-Carotene Bleaching Activity. The β-carotene bleaching activity occurred only when linoleic acid was present in the Lox assay mixture: the effect of linoleate concentration on the disappearance of β-carotene was studied, and the bleaching activity was greatest when linoleic acid was 2.31 mM (data not shown).

Amino Acid Analysis. The amino acid composition of Lox is shown in Table 3. The number of residues of each amino acid was calculated on the basis of a monomer molecular mass of 95 kDa and 7 prolines residues. The data represent the nearest integer to the average of two analyzes. The cysteine residue content was not detectable by the used methods.

The total number of amino acids is thus 858, a value comparable to that found for soybean LOX-1, equal to 839 (Shibata et al., 1987).

Molecular Mass and Subunit Composition. The enzyme molecular mass, determined by gel filtration, was 95 ± 5 kDa. A single band corresponding to a molecular mass of ~95 kDa was obtained by SDS−PAGE, indicating that a monomeric structure can be postulated for the enzyme (data not shown). Our molecular mass determination is in agreement with literature data for plant enzymes (90−100 kDa) (Shibata et al., 1987). It is worth noting that plant enzymes have an N-terminal sequence, which is lacking in animal lipoxygenases having molecular masses in the range of 65−75 kDa (Chen et al., 1994; Prigge et al., 1996; Steczco et al., 1992).

Effect of Temperature. The results indicated that the maximum Lox activity on linoleic acid was at 40 °C with an increase of activity of ~22% with respect to the standard assay at 25 °C. The enzyme was also active at 10 °C; in fact, it shows ~50% of its greatest activity. Lox exhibited also a 75% increase in β-carotene bleaching activity at 60 °C with respect to that at 25 °C; in

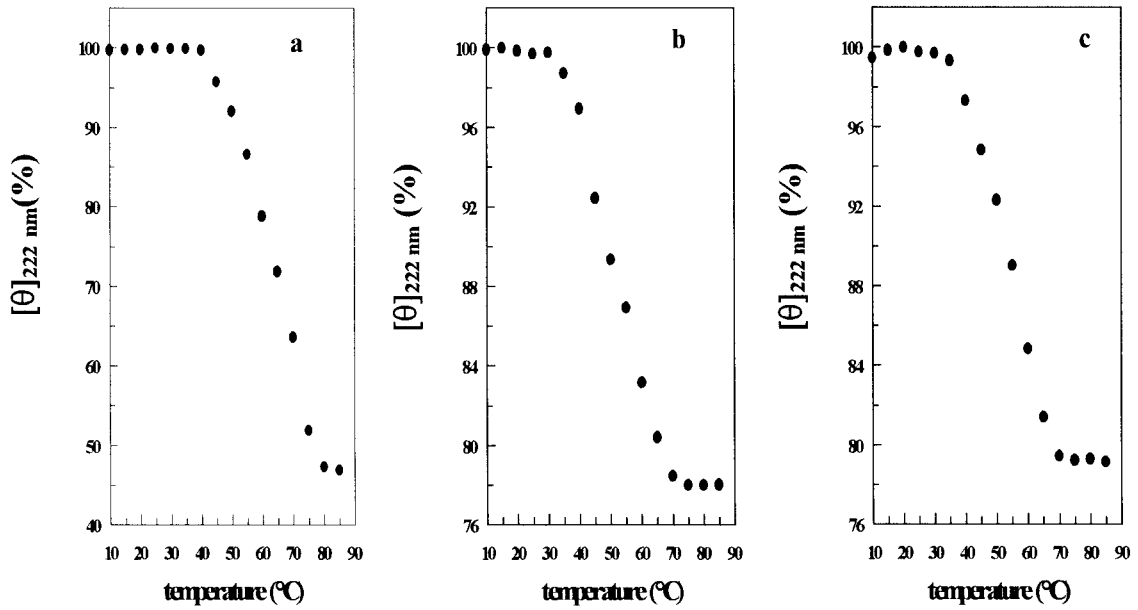

Figure 7. Dependence of Lox α-helix content on temperature by far-UV CD measurements at 222 nm in the temperature range between 10 and 85 °C: (a) at pH 5.0; (b) at pH 6.8; (c) at pH 9.0.

addition, the bleaching activity at 10 °C was 12.5% of its greatest value. These results suggested that during pasta processing the enzyme could cause the loss of carotenoid pigments also at low temperatures (Figure 3).

NH_2-Terminal Sequence. The purified Lox, subjected to the Edman degradation, produced the following amino acid residues:

QSALLVLTVVLMIKNLF

The NH_2-terminal sequence of Lox was searched for homology against the SWISS Prot data bank using the FASTA program. Sequence alignments were performed by the multialignment program PILEUP (default setting) and when necessary by a word processor program.

The results showed a high similarity to several lipoxygenases from different organisms. Figure 4 illustrates the sequence alignment of the NH_2-terminal sequence of our Lox with the NH_2-terminal sequence of lipoxygenases from soybean (isoenzymes 1, 2, 3a, and 3b), potato, common tobacco, *Arabidopsis thaliana* (At-Lox1), kidney bean (phavu), garden pea, and garden pea (pea2): all were determined on the basis of their DNA sequences.

This alignment points out a region of high sequence identity if our Lox is compared with the other lipoxygenases (amino acids in bold). The best sequence homology was observed with lipoxygenase-1 from kidney bean (phavu) (51.3%) (Chen et al., 1994).

Circular Dichroism Studies. Far-UV circular dichroism spectra of Lox recorded between 200 and 240 nm at 25 °C in buffers at pH 5, 6.8, and 9 are shown in Figure 5; we present only that at pH 6.8 because the three spectra are exactly alike. They appear very similar in all of the pH regions investigated showing any perturbation of the secondary structure by these pH values. The protein possesses a secondary structure content rich in α-helices and β-structures calculated according to Yang's algorithm (data not shown).

In Figure 6 are shown the spectra in the near-UV at 25 °C and at pH 5.0, 6.8, and 9.0. The protein spectrum at pH 6.8 appears well structured, suggesting that the aromatic residues are involved in molecular interactions into the protein matrix as pointed out by the maxima at 293, 285, 276, and 260 nm, respectively. Contrary to what was observed in the far-UV, in the near-UV, acidic or basic pH values perturb the tertiary structure of the protein and, as a consequence, we observe a decrease of the dichroic activity at 293 and 285 nm. Moreover, the dichroic activity attributable to phenylalanine and tyrosine residues became negative, suggesting a strong modification of the tertiary architecture of the protein.

The thermal stability of the enzyme investigated at different pH values by measuring the dichroic activity at 222 nm as a function of the temperature is depicted in Figures 7. A sigmoidal decrease depending on increasing temperature was observed. The inflection point was at 60 °C at pH 5.0, whereas it was at 50 °C for pH 6.8 and 9.0. These data show that the protein α-helix content is pH dependent, suggesting that the protein is less stable at these last pH values in the temperature range examined. As the spectra point out, the percentages of the final denaturation states of the protein are different as a consequence of the combined effect of temperature and acidic pH that cause a larger extent of denaturation if compared to the other experimental conditions.

Storage Stability. The Lox storage stability experiments were performed at 4 and 25 °C in the absence and in the presence of 1 mM β-mercaptoethanol, 1 mM dithiothreitol, or 200 ppm of L-ascorbic acid and at 25 °C in absence of effectors. The results showed as expected a better stability of the Lox at 4 °C. In fact, at this temperature the enzyme retained 100% of its activity after 140 h of incubation, whereas at 25 °C (Figure 8) the half-life was only 17 h. A marked inhibition occurred when the enzyme was incubated at 4 °C in the presence of reducing agents such as dithiothreitol and/or β-mercaptoethanol; this was probably caused by hydroperoxide ion produced by Fenton-like reaction as already described for the human 5-Lox (Percival et al., 1992). The addition of L-ascorbic acid to the enzyme mixture caused a rapid inhibition at 25 °C (data not shown) as well as at 4 °C, suggesting a

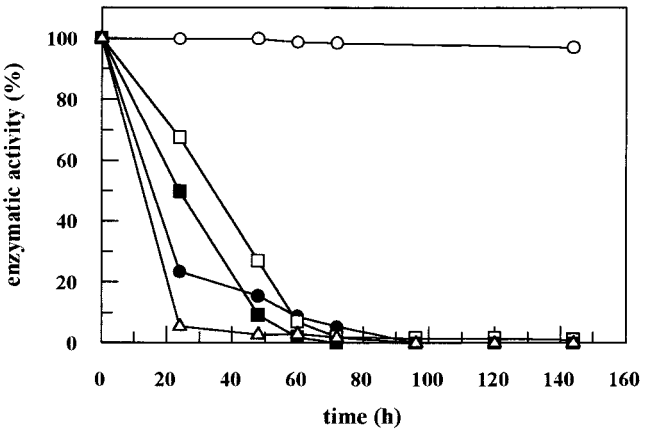

Figure 8. Dependence of Lox storage stability at 25 °C (●) and at 4 °C (○) and in the presence of 1 mM β-mercaptoethanol (■) at 4 °C, 1 mM dithiothreitol (□) at 4 °C, and 200 ppm of L-ascorbic acid (△) at 4 °C.

potential use as a natural enzyme inhibitor to avoid colorless or off-color pigments during pasta processing (Walsh et al., 1970).

CONCLUDING REMARKS

These studies have allowed a fast and high yield purification procedure with the identification of linoleic acid as the substrate of higher specificity as well as the presence of a β-carotene bleaching activity. The different pH maxima exhibited by the enzyme for these two activities can be correlated to the results observed in the near-UV spectra collected at acidic pH showing a different exposition and/or flexibility of the aromatic side chains of the enzyme, so suggesting, as already observed (Hsieh and Mc Donald, 1984), that during the β-carotene bleaching is involved an absorption mechanism of β-carotene on the enzyme that we have rationalized, in the present work, in terms of a different tertiary structure observed at pH 5.0 if compared to that at pH 6.8.

The selection of a cultivar with a low lipoxygenase content or the use of natural inhibitors of this activity in the processing of pasta products from durum wheat semolina can be of great biotechnological relevance to preserve β-carotene and the related pasta quality. In this regard, our current aim is to study gene cloning to reveal the primary structure of this lipoxygenase from durum wheat as well as to describe its detailed physicochemical and enzymological properties as has been done for such enzymes from other sources.

We aim for a complete identification and characterization of the entire gene family that will make it possible to address the means by which specific lipoxygenase genes are regulated, as well to define how the different isozymes influence the overall physiology of the plant.

ABBREVIATIONS USED

Lox, lipoxygenase from durum wheat; HPLC, high-performance liquid chromatography; EDTA, ethylendiaminetetraacetic acid; MESNA, mercaptoethanolsulfonic acid; FPLC, fast protein liquid chromatography.

ACKNOWLEDGMENT

We thank Mr. G. De Simone for his technical assistance and Mrs. Giuseppa Travaglione from the Dpt. Organic and Biological Chemistry, University of Naples, for her technical assistance in the amino acid analysis.

LITERATURE CITED

Anderson, J. M. Membrane-derived fatty acids as precursors to second messengers. In *Second Messengers in Plant Growth and Development*; Boss, W. F., Morre, D. J., Eds.; Liss: New York, 1989; pp 181−212.

Andrews, P. Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **1964**, *91*, 222−223.

Axerold, B.; Cheesbrough, T. M.; Laakso, S. Lipoxygenase from soybeans. *Methods Enzymology* **1981**, *71*, 441−451.

Ben Aziz, A.; Grossman, S.; Ascarelli, I.; Budowski, P. Carotene bleaching activities of lipoxygenase and heme proteins as studied by a direct spectrophotometric method. *Phytochemistry* **1971**, *10*, 1445−1452.

Chen, X.-S.; Kurre, V.; Jenkins, N. A.; Copeland, N. G.; Funk, C. D. DNA cloning, expression, mutagenesis of c-terminal isolucine, genomic structure and chromosomal localizations of murine 12-lipoxygenase. *J. Biol. Chem.* **1994**, *296*, 13979−87.

Gaffney, B. J. *Annu. Rev. Biophys. Biomol. Struct.* **1996**, *25*, 431−459.

Gardner, H. W. Recent investigations into the lipoxygenase pathway of plants. *Biochim. Biophys. Acta* **1991**, *1084*, 221−239.

Hildebrand, D. F. Lipoxygenase. *Physiol. Plant* **1989**, *76*, 249−253.

Hsieh, C. C.; McDonald, C. E. Isolation of Lipoxygenase Isoenzymes from flour of durum wheat endosperm. *Cereal Chem.* **1984**, *61* (5), 392−398.

Irvine, G. N. Durum wheat and pasta products. In *Wheat Chemistry and Technology*, 2nd ed.; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1971; pp 777−797.

Irvine, G. N.; Anderson, J. A. Kinetic studies of the lipoxidase system of wheat. *Cereal Chem.* **1953**, *30*, 247−253.

Kato, T.; Shirano, Y.; Iwamoto, H.; Shibata, D. *Plant Cell Physiol.* **1993**, *34*, 1063−1072.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **1970**, *227*, 680−685.

Leatherbarrow, R. J. Grafit, version 2.0; Erithacus Software: Staines, U.K., 1990.

Lowry, D. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Lulai E. C.; Baker C. W. Physicochemical characterization of barley lipoxygenase. *Cereal Chem.* **1976**, *53*, 777.

Manna, F.; Borrelli, G. M.; Massardo, D. R.; Wolf, K.; Alifano, P.; Del Giudice, L.; Di Fonzo, N. Differential expression of lipoxygenase genes among durum wheat cultivars. *Cereal Res. Commun.* **1998**, *26*, 23−30.

McDonald, C. E. Lipoxygenase and Lutein Bleaching Activity of Durum Wheat Semolina. *Cereal Chem.* **1979**, *56* (2), 84−89.

Percival, M. D.; Denis, D.; Riendeau, D.; Gresser, M. J. Investigation of the mechanism of non turnover dependent inactivation of purified human 5-lipoxygenase. *Eur. J. Biochem.* **1992**, *210*, 107−109.

Prigge, S. T.; Boyington, J. C.; Gaffney, B. J.; Amzel, L. M. Structure conservation in lipoxygenases: structural analysis of soybean lipoxygenase-1 and modeling of human lipoxygenases. *Proteins: Struct., Funct. Genet.* **1996**, *24* (2), 275−291.

Shibata, D.; Steczko, J.; Dixon, J. E.; Hermodson, M.; Yazdanparast, R.; Axelrod, B. Primary structure of soybean lipoxygenase-1. *J. Biol. Chem.* **1987**, *262*, 10080−10085.

Shibata, D.; Steczko, J.; Dixon, J. E.; Andrews, P. C.; Hermodson, M.; Axelrod, B. Primary structure of soybean lipoxygenase L-2. *J. Biol. Chem* **1988**, *263*, 6816−6821.

Siedow, J. N. Plant lipoxygenase: structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145−188.

Steczco, J.; Donoho, G. P.; Clemens, J. C.; Dixon, J. E.; Axelrod, B. Conserved histidine residues in soybean lipoxygenase: functional consequences of their replacement. *Biochemistry* **1992**, *31*, 4053−4057.

Talia, S. A.; Sagi, F. Relationships between chemical composition of Durum Wheat Semolina and macaroni quality. II. Ash, carotenoid pigments and oxidative enzymes. *Cereal Res. Commun.* **1987**, *15* (2−3), 123−129.

Tressl, R.; Bahri, D.; Silwar, R. Determination of aroma substance in Spalter hops by gaschromatography−mass spectrometry. *Z. Lebensm. Unters. Forsch.* **1978**, *166* (6), 350−354.

Van Aarle, P. G. M.; de Barse, M. M. J.; Veldink, J. F. G.; Vliegenthart, J. F. G. Purification of a lipoxygenase from ungerminated barley. *FEBS Lett.* **1991**, *280* (1), 159−162.

Verhue, W. M.; Franke, A. The heterogeneity of soyabean lipoxygenase. Unilever Research, Vlaardingen (The Netherlands). *Biochim. Biophys. Acta* **1972**, *284* (1), 43−53.

Vick, B. A.; Zimmerman, D. C. In *The Biochemistry of Plants. A Comprehensive Treatise*; Stumpf, P. K., Conn, E. E., Eds.; Academic Press: New York, 1987; Vol. 9, pp 53−90.

Walsh, D. E.; Youngs, V. L.; Gilles, K. A. Inhibition of Durum Wheat Lipoxygenase with L-Ascorbic Acid. *Cereal Chem.* **1970**, *47* (2), 224−230.

Yang, G.; Schwarz, P. B.; Vick, B. A. Purification and Characterization of Lipoxygenase Isoenzymes in Germinating Barley. *Cereal Chem.* **1993**, *70* (5), 589−595.

Yenofsky, R. L.; Fine, M.; Liu, C. Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3 gene. *Mol. Gen. Genet.* **1988**, *211*, 215−222.

Received for review July 30, 1998. Revised manuscript received February 8, 1999. Accepted March 8, 1999. Research was supported by the Ministero delle Risorse Agricole, Alimentari e Forestali (MiRAAF), Italy, a special grant "MICIA" to L.D.G. and N.D.F. This research was also supported by European Community Grant FESR 94.05.09.013 and by National Research Council (CNR-Roma).

JF980853Z