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Changes in Lipoxygenase Properties and Activity Related to Postgerminative Growth and Senescence in Oat (*Avena sativa* L. cv. Victory 1)

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Lipoxygenase (LOX), one of the main oxidative catalysts in plants, is involved in the regulation of growth and senescence. We investigated changes in LOX activity or its properties as they related to the development of oat plants at four stages (germination, growth, natural senescence, and dark-incubated senescence). LOX activity was high during early growth and at senescence. At pH 4.5, activity showed an abrupt surge compared with a normal enzyme reaction pattern at pH 6.5. The optimum reaction temperature was 25°C; LOX and peroxidase exhibited similar activity patterns. Polyacrylamide gel electrophoresis revealed that the purified LOX consisted of three isoenzymes in germinating seeds, two in growing seedlings, and three during both natural and dark-induced senescence. As determined by isoelectric focusing, the isoelectric points (pI) of LOX ranged from 3.6 to 6.5 throughout the four developmental stages; for natural or dark-induced senescence, the pI was 9.0.

Keywords: isoelectric focusing, lipoxygenase, oats, polyacrylamide gel electrophoresis, seedlings, seeds

Lipoxygenase (linoleate: oxygen 13-oxidoreductcase, EC 1.13.11.12) is one of the main oxidative catalysts in plants. Also known as LOX and carotene oxidase, it catalyzes the stereo-specific dioxygenation of polyunsaturated fatty acids containing *cis*-1,4-pentadiene structures, leading to the formation of various hydroperoxide derivatives (Axelrod et al., 1981; Vick and Zimmerman, 1983; Siedow, 1991; Rosahl, 1996; Brash, 1999). These products function as growth regulators, antimicrobial compounds, and signal molecules, as well as providing flavors and aromas (Rosahl, 1996). As a highly reactive species (Vick and Zimmerman, 1987), the LOX reaction also leads to the formation of superoxide radicals (Lynch and Thompson, 1984).

Generation of these highly reactive species supports the hypothesis that LOX is involved in cell senescence and pathogen defenses (Thompson et al., 1987; Hildebrand et al., 1988; Siedow, 1991). LOX may also play roles in membrane turnover and various other cellular and developmental processes (Hildebrand et al., 1988; Siedow, 1991), including 1) LOX reactions that provide substrates for other metabolically important enzymes, 2) regulation of plant growth and development, and 3) degradation during senescence, wounding, and infection, all of which involve membrane breakdown (Mack et al., 1987). In plants, the most common substrates for LOX are linoleic acid and linolenic acid, two major fatty acids found in the thylakoid membrane (Vick and Zimmerman, 1987). LOX is also located in the cytosol (Siedow, 1991), chloroplasts (Feussner et al., 1995), and vacuoles (Wardale and Lambert, 1980).

Feussner and Wasternack (2002) have reported that the biosynthesis of jasmonic acid (JA) is initiated by the insertion of molecular oxygen into Position 13 of a-linolenic acid catalyzed by a 13-lipoxygenase. JA is an important terminal product of the LOX pathway (Vick and Zimmerman, 1983, 1984, 1987; Feussner and Wasternack, 2002; Hause et al., 2003; Mizuno et al., 2003).

Multiple forms or isoenzymes of LOX have been detected in *Arabidopsis* (Bell et al., 1995), barley (Voros et al., 1998), tomato (Heitz et al., 1997), potato (Geerts et al., 1994; Royo et al., 1996, 1999), and maize (Jensen et al., 1997; Kim et al., 2003). The most extensively studied plant LOXs have been from soybean seeds (Tranberger et al., 1991; Dubbs and Grimes, 2000; Narvel et al., 2000), where two forms

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have been classified: Type 1, with a basic optima at pH 8 to 9; and Type 2, those with a neutral pH optima (Vick and Zimmerman, 1987). Saravitz and Siedow (1995) have divided LOX from soybean leaves into two distinct groups: those with neutral pIs ranging from pH 6.8 to 7.2, and those with acidic pIs of pH 4.7 to 5.6. Kiran Kumar et al. (1992) have screened various plant sources for LOX activity, and have found that most lipoxygenases exhibit relatively high activities at pH 6.0, suggesting an acidic nature. In mature soybean seeds, three to four LOX isoenzymes are present, and three additional isoenzymes appear in the soybean cotyledons during germination. Their roots and hypocotyls have LOX isoenzymes distinct from those found in the seeds, whereas the leaves possess three distinctive isoenzymes (Grayburn et al., 1991). Likewise, four isoenzymes have been identified in pea seeds (Yoon and Klein, 1979). Although multiple isoenzymes for LOX are found in many plant species (Christopher et al., 1970; Mulliez et al., 1987; Todd et al., 1990), their physiological role has yet to be established.

Our objective was to understand more fully the role of LOX in the development and degradation processes of oat plants, and to determine the number of LOX isoenzymes at various developmental stages, from germination through senescence.

MATERIALS AND METHODS

Plant Materials

Oat seeds (*Avena sativa* var. L. cv. Victory 1; from Allmanna Svenska Utasades A.-B., Sweden) were de-husked, surface-sterilized with 0.5% NaOCl for 10 min, thoroughly washed with distilled water, and sown at a depth equal to their diameter in pots (50 × 25 × 6 cm) filled with TERRA-LITE Vermiculite (Scotts-Sierra Horticultural Products, USA). They were well-watered, but without additional nutrients, to avoid any fertilizing effects. The seedlings were reared in a growth chamber for 7 to 11 d, at a relative humidity of 65 to 75%. The chamber was controlled with a 14-h photoperiod, at a light intensity of 200 $E \cdot m^{-2} \cdot s^{-1}$, 24 to 26°C (daytime), and 10 h at 10°C during the night cycle.

LOX properties were investigated during four developmental stages: 1) seeds at 3 d after sowing (DAS) (germination); 2) seedlings at 5 DAS (growth); 3) seedlings at 10 DAS (senescence); and 4) seedlings at 10 DAS (artificial senescence, induced by transferring seedlings at 7 DAS to dark conditions for 3 d).

Growth Rate

Shoot lengths (cm) were measured each day on 10+ seedlings, from 3 DAS to 11 DAS. Their growth rate (cm day^{-1}) was defined as the increase in length from one day to the next.

pH Profile of Crude Extracts

Linoleic acid (Sigma, USA) solutions (substrate, 2.07 mM) buffered at various pH levels were used to determine the optimum pH of the LOX enzymes. Measurements were made at various pHs (3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, and 9.0) with 50 mM mixing buffer that consisted of Tris-HCl, sodium phosphate, and citric acid.

Purification of Lipoxygenase (LOX) and Spectrophotometric Assay

Germinating seeds and seedlings (20 g) were ground to powder in liquid nitrogen, then homogenized in a mortar at 4°C in 50 mM sodium phosphate buffer (pH 6.5). Extracts were centrifuged for 15 min at 12,000g. After centrifugation, the precipitate was discarded and the supernatant was added with $(NH_4)_2SO_4$ by stirring. The precipitate between 30% and 60% saturation was collected and applied to a Sephadex G-50 column, which had previously been equilibrated with 50 mM sodium phosphate buffer (pH 6.5). Fractions of 3 ml were collected at a flow rate of 50 ml h^{-1} and assayed for protein and LOX activity. Those that exhibited LOX activity were combined, and $(NH_4)_2SO_4$ was added. The precipitate between 30% and 60% saturation was applied to a Sephadex G-200 column under similar conditions. Fractions exhibiting LOX activity were pooled, concentrated by ultrafiltration (Centricon YM-10, Millipore, USA), and stored at -80°C.

LOX activity in the leaf extracts was measured at 234 nm according to the method of Ben-Aziz et al. (1970), using a spectrophotometer (8452 A Diode Array Spectrophotometer, Hewlett Packard, USA) equipped with HP 89531 A MS-DOS-UV/VIS operating software. The substrate was 0.25% Tween 20 in 50 mM Tris-HCl and 2.07 mM linoleic acid. A 100-μL aliquot of Tween 20 and the linoleic acid was added to a 1.0-mL cuvette containing 1 mL of 50 mM mixing buffer -- Tris-HCl, sodium phosphate, and citric acid (pH 4.5). Then, 10 μL of the extract solution was

added to the cuvette to assay for LOX activity (Romero and Barrett, 1997). Changes in absorbance at 234 nm (25°C) were monitored; the reaction was linear for at least 10 min. One unit was defined as the amount of enzyme that produced a change in absorption of 0.001 min^{-1} at 234 nm; this corresponded to a catalytic activity of 0.667 nkat (absorption coefficient of the peroxide = 25,000 M^{-1} cm^{-1}) (Ocampo et al., 1986).

Peroxidase Assay

Plant samples were homogenized in liquid nitrogen to measure peroxidase activity, using hydrogen peroxide as the substrate (Maehly and Chance, 1954; Chance and Maehly, 1955). The plant materials were extracted with 10 mM phosphate buffer (pH 6.0). After centrifugation under 1000*g* at 4°C for 10 min, the supernatant was used to determine soluble peroxidase activity. In all, 2.9 mL of substrate stock solution (including 0.003% hydrogen peroxide and 0.01 M phosphate buffer at pH 6.0) was added to a cuvette containing 25 µL of 1% *o*-dianisidine in methyl alcohol (Maehly, 1955; Lee and Lin, 1996). Approximately 75 µL of extract was then added to the cuvette. The change in absorbance at 460 nm was monitored for 1 min, and the rate of change per minute was calculated (Chmiélnicka et al., 1971). One unit of peroxidase activity was defined as the amount of enzyme that decomposed 1.0 mm of peroxide per minute. The molar absorbance of hydrogen peroxide is equal to 1.13×10^4 $M^{-1}cm^{-1}$ at 460 nm.

Protein Measurements

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin (BSA, Fermentas, USA) as the standard. Total protein in the leaf extracts was calculated with a protein standard and Bio-Rad protein assay that was based on the dye-binding assay of Bradford (1976).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a minigel apparatus (70 × 80 × 0.75 mm) according to the system of Laemmli (1970), using 7.5% acrylamide running and 4% stacking gels. The protein gel was run at 4°C for 30 min at 50 V, and for 60 min at 100 V. Protein bands were stained by immersing the gels in a 0.05% (w/v) Coomassie Brilliant Blue R-250 (Fermentas) solution, in 45% methanol, and in a 9% acetic acid solution.

Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) was carried out in a minigel apparatus (90×60×0.4 mm). Native IEF was done in a 6% polyacrylamide gel that included 2% (v/v) 3/10 ampholine (Pharmacia, USA). The focusing was run at 4°C and 150 V for 30 min, then 200 V for 12 h.

Statistical Analysis

This study had a replicated experimental design. Analyses of variance (ANOVA) were performed to test the hypothesis that various LOX isoenzymes would be detected at different pHs for each developmental stage. A Proc GLM test from the SAS (2001) program was used to obtain p-values.

RESULTS

Relationship between Growth and LOX Activity

Oat seedlings were measured from the ground to their tops, for 3 to 11 d after sowing (DAS; Fig. 1). Their overall growth rate, defined as the daily change in shoot length, was highest at 5 DAS (3.59 cm d^{-1}) but then sharply declined. After 9 DAS, the seedlings exhibited symptoms of apparent senescence, e.g., an absence of extension and a gradual loss of green coloring and leaf expansion. For this study, therefore, seedlings at 5 DAS were chosen as the model for the active growth stage while those at 10 DAS repre-

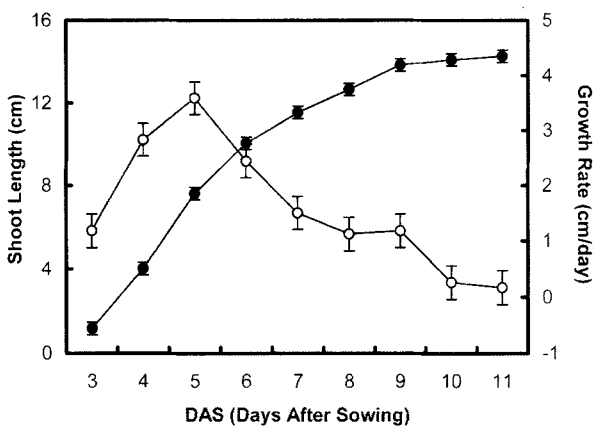

Figure 1. Shoot lengths (●–●) and growth rates (○–○) of oat seedlings after germination. Each value represents mean of 10 plants; bar indicates SE, $p<0.05$.

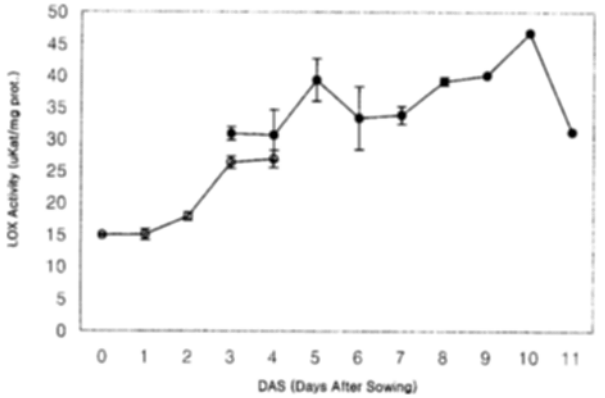

Figure 2. Changes in LOX activity of oat seeds (○–○) and seedlings (●–●) measured spectrophotometrically at 234 nm using 2.07 mM linoleic acid as substrate (pH 4.5). Each value represents mean ± SE of three independent experiments. Differences in activity between days are $p<0.05$.

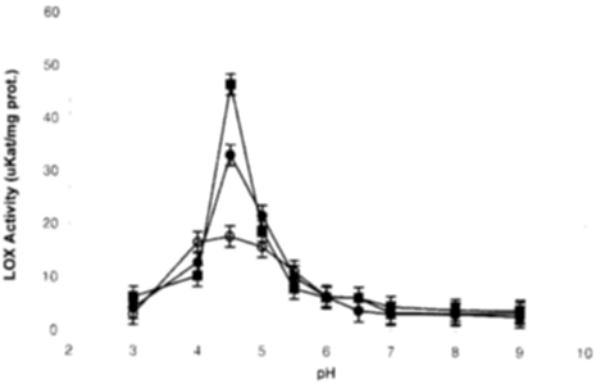

Figure 3. pH profiles of LOX activity in crude extracts of oat plants measured spectrophotometrically at 234 nm. (○–○) seeds at 3 days after sowing (DAS); (●–●) 5 DAS; (■–■) 10 DAS. Each value is mean ± SE of independent triple experiments. LOX activity and pHs relationships are $p<0.05$, $n=3$.

sented natural senescence.

LOX activity measured at pH 4.5 was the highest of all enzymes up to 11 DAS (Fig. 2). Its LOX specific activity was greater during the growth stage than at germination. For the latter, activity increased little until 2 DAS (17.9 mKat mg^{-1} protein), followed by a significant increase at 3 DAS (26.5 mKat mg^{-1} protein), where it remained unchanged until 4 DAS. At 3 and 4 DAS, LOX activity was higher in the seedlings than in seeds compared at the same DAS (30.9 vs. 26.5 mKat mg^{-1} protein, $p<0.05$, Fig. 2). In the growth phase, two activity peaks were detected: one at 5 DAS (39.4 mKat mg^{-1} protein), the other at 10 DAS (46.8 mKat mg^{-1} protein). These results coincide well with the growth rate pattern (Fig. 1), which showed its highest peak at 5 DAS and its greatest decline at 10 DAS. The former peak may have indicated an active growth status following a previous lag phase (3 and 4 DAS) while the latter suggested an active senescence phase.

pH Dependence of LOX Activity

The pH optima for LOX activity in seeds occurred at 3 DAS; in seedlings, at 5 DAS and 10 DAS (Fig. 3). All stages had their highest specific LOX activity at pH 4.5. The only differences were in absolute peak activities (26.5 mKat mg^{-1} protein at 3 DAS, 39.4 mKat mg^{-1} protein at 5 DAS, and 46.8 mKat mg^{-1} protein at 10 DAS). Among the three crude extracts, seedlings in the senescence stage (10 DAS) had the highest specific activity (Fig. 2).

Purification of Oat LOX

Data from a typical purification, using the extract from seedlings at 5 DAS, are summarized in Table 1. Following the Sephadex G-200 column phase, the enzyme was purified about 4-fold, with a 33% yield. This result was comparable with that obtained from a 6-fold purification of grape LOX on phenyl Sepharose CL-4B resin (Zamora et al., 1985). Elution profiles were achieved with partially purified LOX from four developmental stages on a Sephadex G-200 column (Fig. 4). LOX activity was measured at pH 4.5, 6.5, and 8.5, but could not be observed at the highest pH (data not shown). All of these extracts had a peak of activity at pH 4.5 on column Fraction 4 and at pH 6.5

Table 1. Purification of LOX from oat seedlings of 5 DAS (based on 10 g fresh weight).

Step of purification	Protein (mg)	Total activity (μKat)	Specific activity (μKat/mg protein)	Purification (fold)
Extracts	150.0	6670.0	440.0	1.0
Ammonium sulfate fraction, 30-60%	88.0	6140.0	700.0	1.6
Sephadex G-50	23.4	3600.0	1540.0	3.5
Sephadex G-200	10.1	2120.0	2140.0	4.9

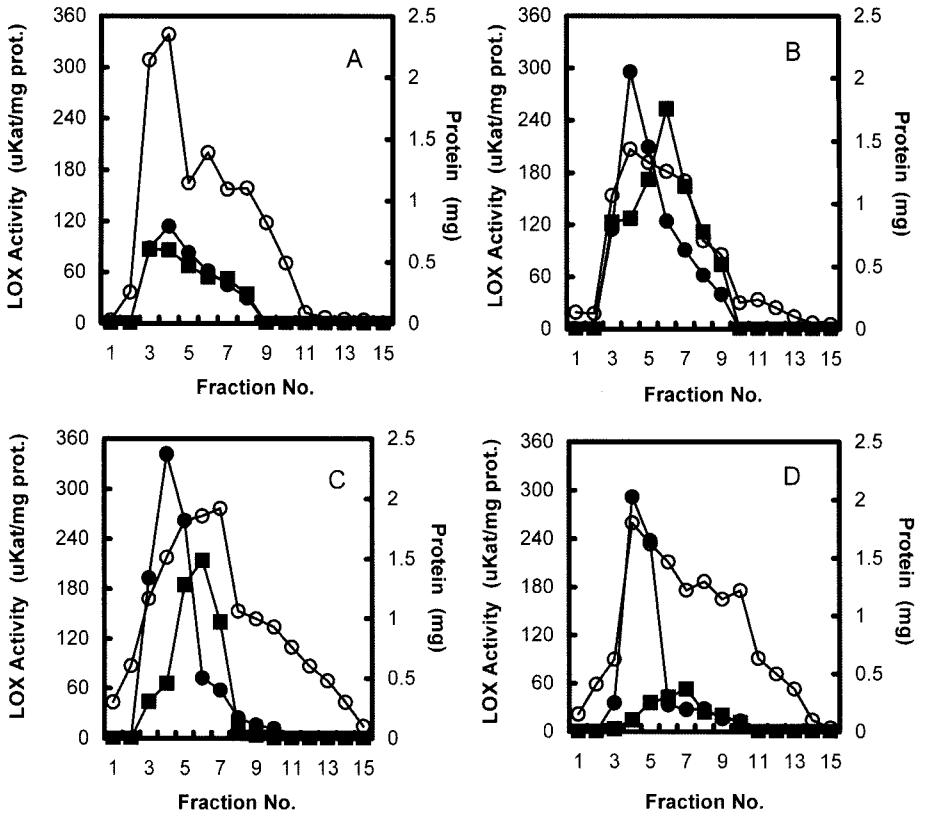

Figure 4. Elution profile of oat lipoxygenase on Sephadex G-200 column. Flow rate was regulated at 50 mL h^{-1} and 3-mL fractions were collected. (○–○) Protein, absorbance at 280 nm; (●–●) lipoxygenase activity at pH 4.5; (■–■) lipoxygenase activity at pH 6.5.

on column Fraction 6. LOX activity at pH 4.5 changed according to the developmental stage.

During germination (3 DAS; Fig. 4A), LOX activity was low, ranging from 120 mKat mg^{-1} protein (pH 4.5) to 80 mKat mg^{-1} protein (pH 6.5). Seedlings at 5 DAS contained 300 mKat mg^{-1} protein at pH 4.5, and 260 mKat mg^{-1} protein at pH 6.5 (Fig. 4B). By 10 DAS, activity had increased to 350 mKat mg^{-1} protein at pH 4.5, but was only 210 mKat mg^{-1} protein at pH 6.5 (Fig. 4C), compared with levels measured in the germinating seeds (Fig. 4A). When seedlings at 5 DAS were incubated in the dark for 3 d, their LOX activity increased 3-fold (300 mKat mg^{-1} protein; Fig. 4D), compared with those at the germinating stage. How-

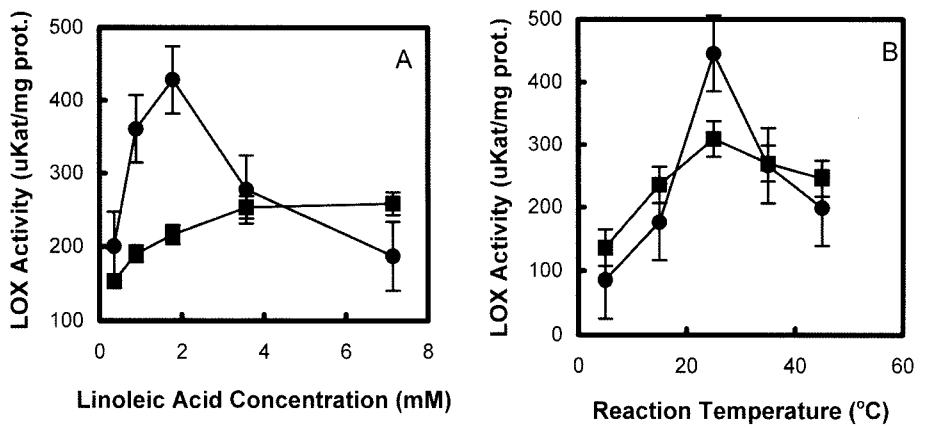

Figure 5. Purified oat LOX activity at different linoleic acid (substrate) concentrations **(A)** and reaction temperatures **(B)**. (●–●) LOX activity at pH 4.5; (■–■) LOX activity at pH 6.5. Values represent mean ± SE of three measurements, $p<0.05$.

Table 2. Lipoxygenase and peroxidase activities in oat seeds and seedlings. LOX activity was measured using 2.07 mM linoleic acid as substrate at pH 4.5. Each activity was based on 1 g fresh weight. Values are means ± SE; n=3, $p<0.05$. DAS (days after sowing). **A**, seeds at 3 DAS; **B**, seedlings at 5 DAS; **C**, seedlings at 10 DAS; **D**, seedlings at 10 DAS treated in dark for 3 d after 7 DAS.

Stage	Lipoxygenase activity		Peroxidase activity		Protein (mg)
	Total (μKat)	Specific (μKat/mg protein)	Total (units)	Specific (units/mg protein)	
A	460.3 ± 5.7	202.7 ± 8.8	9.0 ± 0.005	1.07 ± 0.03	2.27 ± 0.04
B	268.5 ± 4.3	216.5 ± 12.1	5.5 ± 0.006	1.23 ± 0.06	1.24 ± 0.05
C	413.6 ± 6.0	236.3 ± 12.0	9.5 ± 0.008	1.44 ± 0.06	1.75 ± 0.08
D	326.8 ± 12.6	245.7 ± 33.5	8.0 ± 0.003	1.65 ± 0.03	1.33 ± 0.03

ever, LOX activity at pH 6.5 was the lowest (50 mKat mg^{-1} protein) among the four stages assessed here. Clear differences were found between activities at pH 4.5 and pH 6.5 (Fig. 4). As the oat seedlings matured, these differences were enhanced. Likewise, our dark-incubated seedlings showed maximum differences among the various pH levels. We can infer, according to particular stages, that multiple LOX isoenzymes develop differently and separately (independently), based on the physiological state of the plants. For example, during germination, many types of enzymes appeared to participate in seedling growth. In contrast, during senescence, a stage related to lipid peroxidation of the membranes, LOX played a major role especially under acidic conditions (pH 4.5), and contributed more to lipid peroxidation than at the near-neutral pH of 6.5.

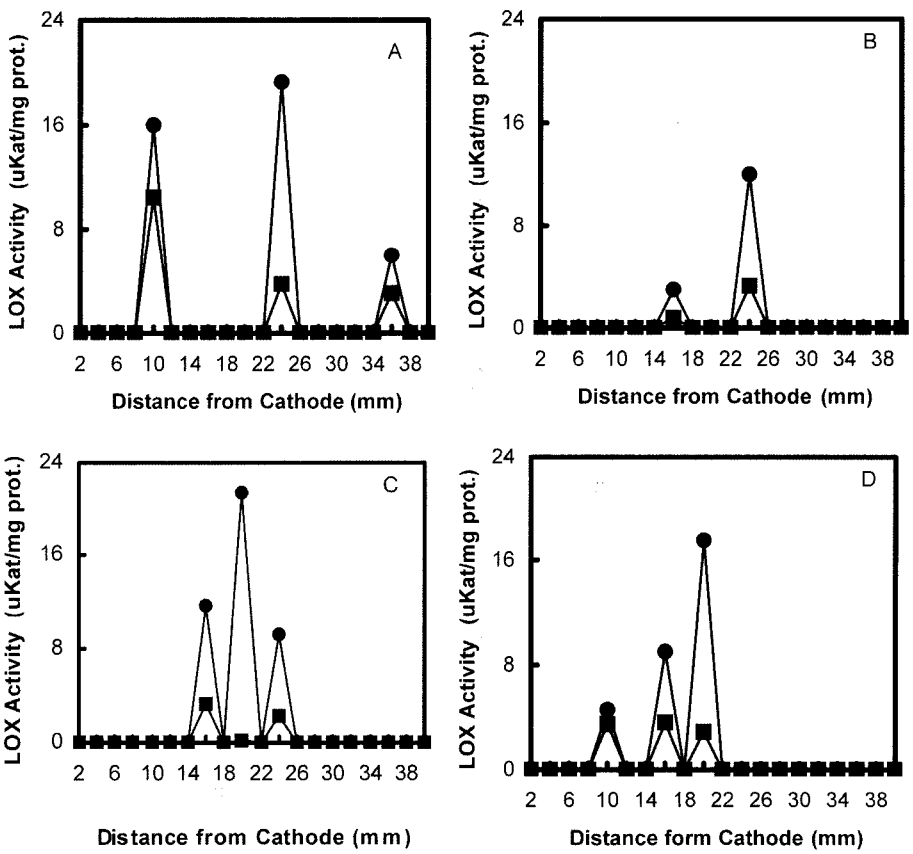

Figure 6. PAGE profiles. (●–●) Lipoxygenase activity at pH 4.5; (■–■) lipoxygenase activity at pH 6.5. **A**, seeds at 3 DAS; **B**, seedlings at 5 DAS; **C**, seedlings at 10 DAS; **D**, seedlings at 10 DAS treated in dark for 3 d after 7 DAS.

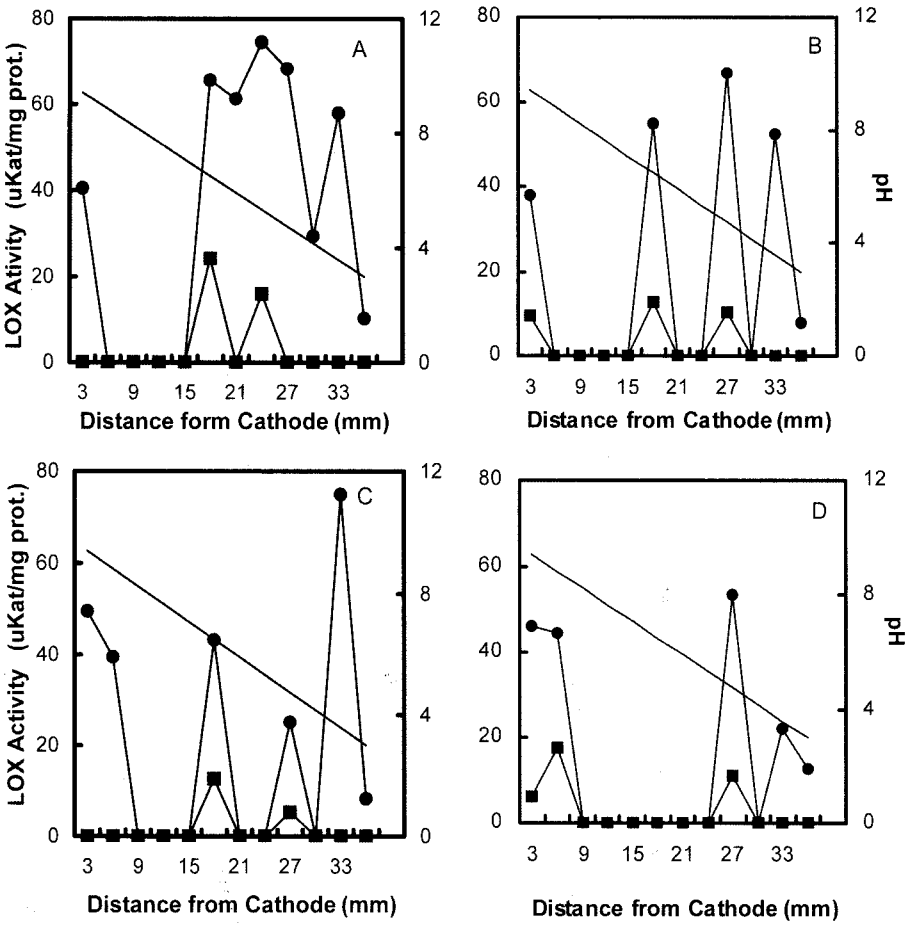

Figure 7. IEF profiles. (●–●) lipoxygenase activity at pH 4.5; (■–■) lipoxygenase activity at pH 6.5; (–) pH gradient. **A**, seeds at 3 DAS; **B**, seedlings at 5 DAS; **C**, seedlings at 10 DAS; **D**, seedlings at 10 DAS treated in dark for 3 d after 7 DAS.

Properties of LOX

For partially purified extracts from 5 DAS, LOX activity at pH 4.5 did not show a normal enzyme reaction pattern (Fig. 5). At 2.07 mM linoleic acid (saturation point), activity increased up to 440 mKat mg^{-1} protein, followed by an abrupt decline to 260 mKat mg^{-1} protein at 3.5 mM linoleic acid. The enzyme-substrate saturation point that occurred at pH 6.5 was 300 mKat mg^{-1} protein and 3.5 mM linoleic acid; 25°C was the reaction temperature at which enzyme activities were optimum for both pH 4.5 and 6.5. At a neutral pH, acidic enzyme activity was influenced less by the reaction temperature.

Relationship between LOX and Peroxidase

For both LOX and peroxidase, specific activities were lowest in seeds at 3 DAS and highest in dark-incubated seedlings (Table 2). With the latter, total LOX activity actually decreased but the decline in protein levels resulted in an increase in LOX activity.

LOX and Its Isoenzymes

Partially purified extracts were collected from column Fractions 3 to 7 on the Sephadex G-200 column, and run on PAGE to identify the isoenzymes for oat LOX (Fig. 6). Because the staining requires a very large quantity of enzyme, activity could not be detected here. However, three distinct LOX bands were present in the germinating seeds (Fig. 6A), with activity at 10, 24, and 36 mm from the gel well (Rf = 0.25, 0.6, and 0.9, respectively) being prominent at pH 4.5, and somewhat lower at pH 6.5. Seedling extracts from 5 DAS (Fig. 6B) revealed one major band at 24 mm (Rf = 0.6), but one minor band at 16 mm (Rf = 0.4) for pH 4.5 and two minor bands for pH 6.5. Seedlings at 10 DAS (Fig. 6C) showed three

major bands at 16, 20, and 24 mm (Rf = 0.4, 0.5, and 0.6, respectively) for pH 4.5 and two minor bands for pH 6.5. Those treated in the dark for 3 d exhibited one major LOX band at 20 mm (Rf = 0.5), two minor bands at 10 and 16 mm (Rf = 0.25 and 0.4; pH 4.5;), and three minor bands for pH 6.5 (Fig. 6D). Whereas the germinating seeds appeared to possess three isoenzymes, seedlings at 5 DAS and 10 DAS had two and three isoenzymes, respectively; dark-incubated seedlings at 10 DAS had three as well. Therefore, we conclude that multiple LOX isoenzymes of different molecular weight exist in oat seedlings.

We performed IEF to identify individual isoenzymes by Isoelectric Point (pI). All four extracts exhibited higher activities in buffer at pH 4.5 than at pH 6.5 (Fig. 7). In 3 DAS germinating seeds (Fig. 7A), high LOX activity at pH 4.5 approached within 18 to 27 mm from the cathode, and pIs ranged from 4.1 to 6.5. The pI of LOX activity for seeds also was shown at 3.5. In seedlings at 5 DAS and 10 DAS (Fig. 7B, C), activities (pIs) were observed at 6.5 and 4.6. For dark-incubated seedlings, two peaks (pIs) were noted at 4.6 and 3.6, while normal seedlings at 10 DAS (Fig. 7C) and dark-incubated seedlings (Fig. 7D) manifested one peak (pI) each at 9.0. Therefore, we conclude that oat seedlings have three isoenzymes with pIs at 6.5, 4.6, and 3.6.

DISCUSSION

LOX Activity

We investigated possible relationships among developmental stages, growth rates, and fluctuations in LOX activity for germinating oat seeds and seedlings. Activity steadily increased during early seedling growth, and then again during early senescence, which occurred from 7 to 10 d after sowing. Specific activity was higher during the growth phase (Fig. 2). In germinating seeds, activity rose only slightly until 2 DAS, then increased significantly at 3 DAS and remained unchanged until 4 DAS. A lag period of 2 d was apparent for LOX activation in those seeds.

During the 7 d following germination, protein contents in the developing leaves declined drastically when no nutrients were added to the seedlings (data not shown). Such a germination-associated increase in LOX activity has been reported in barley (Yabuuchi, 1976), presumably due to *de novo* synthesis of that enzyme in active tissues (Yabuuchi and Amaha, 1975). Soybean seeds are also a rich source of LOX (Axelrod et al., 1981), with activity increasing upon germination (Hilderbrand and Hymowitz, 1983). Luali and Baker (1975) have demonstrated a 2-fold increase in specific LOX activity from whole barley during its 5-d germination period, which they believe is associated with a decline in soluble protein and a concomitant rise in total LOX activity. LOX specific activity is at its maximum in dry chick pea seeds, suggesting its essential role during germination (Sanz et al., 1992). However, the reverse has been reported in sunflower (Belver and Donaire, 1983). Likewise, soluble petal extracts from carnation and gladiolus peak in their LOX activity at the early to mid-senescence stages (Peary and Prince, 1990; Rouet-Mayer et al., 1992). Paliyath and Droillard (1992) have suggested that the earlier action of LOX is related to changes in the structure of the plasma membrane and endoplasmic reticulum.

Based on our profiles of crude extracts over time, LOX activity was consistently highest at an acidic of pH 4.5 during the germination, growth, and senescence stages, with a small peak also being detected at pH 6.5 for seedlings at 5 DAS (Fig. 3). However, contradictory results have been reported with other species. For example, the optimum LOX activity is at pH 6.0 for sweet corn (Romero and Barrett, 1997), pH 5.5 for potato tubers (Mulliez et al., 1987), and pH 7.5 for purified LOX from barley grains (Yabuuchi and Amaha, 1975). In senescing carnation petals, soluble LOX has an optimum pH range of 4.9 to 5.8, with microcosmal LOX exhibiting its maximum activity at pH 6.1 (Rouet-Mayer et al., 1992).

In our study, the slight variations in LOX activity presented in Tables 1 and 2 may have resulted because the quantity of leaf material used was not consistent among experiments.

LOX Purification and Properties

The specific activity of partially purified LOX was 4.9-fold higher than in the crude extract (Table 1). Mulliez et al. (1987) have isolated and purified one major LOX from potato tubers with 3- to 4-fold purifications, while Yabuuchi and Amaha (1975) have purified 10-fold greater LOX from barley grains on a Sephadex G-200 column.

Here, a linoleic acid concentration of 2.07 mM was used in the subsequent assay of LOX activity because it approximated the most common substrate concentration for all methods. LOX exhibited high activity at pH 4.5 through all four developmental stages whereas,

at pH 6.5, activity mainly appeared in the growth and natural senescence stages. Therefore, we infer that at least two different LOX isoenzymes exist in oat plants. Using a DEAE-Trisacryl column, Mulliez et al. (1987) have demonstrated that potato tubers contain at least three LOX isoenzymes. Romero and Barrett (1997) have found that LOX activity from sweet corn is highest at 2.00 mM linoleic acid, at both pH 6.6 and 7.0. Furthermore, Lagocki et al. (1976) has used kinetic analysis of soybean LOX on linoleic acid to propose that, because LOX is a dimeric enzyme, there are two equivalent sites for activation and catalysis.

The Relationship between LOX and Peroxidase

To understand the relationship between LOX and peroxidase during different developmental stages, we used purified extracts for both enzymes. The specific activity for each was lowest in seeds at 3 DAS and highest in dark-incubated seedlings (Table 2). The higher specific activity during senescence may have been caused by the characteristic, active decomposition of membranes. Fatty acid peroxides, which are by-products of a LOX reaction, can subsequently become substrates for peroxidase (Koljak et al., 1997; Brash, 1999). Both enzymes share the pathway, and it is technically difficult to distinguish between the two (Siedow, 1991; Brash, 1999).

Borrell et al. (1997) have reported that, after 6 or 24 h, LOX activity is reduced by approximately 25% in dark-treated oat leaves compared with those incubated in the light. Our study showed an increase in LOX specific activity in the dark-incubated leaves. Although total LOX activity decreased at a rate similar to that reported by Borrell et al. (1997), the decline in protein resulted in greater LOX activity. Here, LOX and peroxidase, closely related to each other, followed the same pattern of activity (Table 2). Chandru et al. (2003) have shown that peroxidase in rice (*Oryza sativa*) is differently activated, depending on the growth stage. Furthermore, dark-grown mung bean seedlings have lower LOX activity than those reared under lights (Zimmerman and Vick, 1970). Therefore, the increase in LOX specific activity in our dark-incubated oat seedlings may have been more a result of lower protein content than a drop in total activity.

Partially purified LOX was run on PAGE, and the four developmental stages had at least one enzyme in common, with germination-associated and senescence-related LOX isoenzymes clearly detected in all extract types. Variations in LOX molecular weights during the stages of development would have been due either to differently processed forms of the same polypeptide gene product or to distinct gene products. On IEF, many isoenzymes had acidic pIs ranging from 3.6 to 6.5. Isoenzymes, including those assayed from the senescing stages, i.e., 10 DAS seedlings and those that were dark-incubated, were clearly evident at pH 9.0. Grayburn et al. (1991) have used chromato-focusing to identify three distinct peaks of activity for soybeans, with pIs of 6.67, 5.91, and 5.67. Therefore, because the isoenzymes isolated from our oat seedlings differed from LOXs obtained from the seeds, we conclude that LOX isoenzymes play a broad role in cell metabolism and disintegration, as well as meeting the various demands for plant growth and development.

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