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TWO LIPOXYGENASE ISOENZYMES AND AN ACTIVATOR IN WHEAT GERM

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; lipoxygenase activator; purification; pH sensitivity.

Abstract—Two isoenzymes of lipoxygenase have been separated and purified from wheat germ. One isoenzyme was stable under both acid and basic conditions. The other isoenzyme was unstable in alkaline solutions and appeared to separate into two electrophoretically distinct active forms. The reaction rate of the isoenzymes towards linoleic acid appeared to be influenced differently as substrate concentrations were increased. A protein fraction extracted from wheat germ activated wheat and soybean lipoxygenase. The effect of the activator may have been to alter the structure of the substrate to enhance reaction rate.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) is found in wheat mill fractions including relatively small quantities in flour [1–3]. Its function, if any, in bread doughs is uncertain [4, 5]. Soybean lipoxygenase, when incorporated in the form of soy flour into wheat bread doughs, has been found to markedly improve the development of the doughs [6], yet soy flour inclusion in wheat flour dough caused a reduction in the quantity of free linoleic acid oxidized [7]. Because bread doughs are generally below the pH range for activity of lipoxygenase 1 from soybean [8], the explanation was offered that lipoxygenase 2, capable of oxidising linoleic acid in both the free and esterified form, may have been competing with the wheat lipoxygenase for the limiting amounts of available oxygen in the dough [7]. Soybean lipoxygenase 2 has recently been implicated in the development of bitter flavours in soy products [9].

Soybean lipoxygenase has been extensively studied, but the properties of wheat lipoxygenase are not well documented [10–12]. A more complete characterization of wheat lipoxygenase seems warranted to help clarify its role in the oxidation of fats in bread dough. Towards this end we report a method for separating two isoenzymes from wheat and for extensively purifying these isoenzymes. In addition, a factor is described which activated the oxidation of linoleic acid by lipoxygenase. This activator was separated from wheat germ extract during lipoxygenase purification.

RESULTS

Preparation and properties of lipoxygenase 1 and 2

Ammonium sulfate fractionation and isoenzyme separation. Attempts to separate lipoxygenase from wheat germ extracts by fractional precipitation with $(NH_4)_2SO_4$ were unsuccessful. When the $(NH_4)_2SO_4$ precipitate containing lipoxygenase was redissolved in buffer, an unidentified flocculent material co-precipitated with lipoxygenase over a period of days. Therefore, fractional solubilization of lipoxygenase from an $(NH_4)_2SO_4$ precipitate was employed. No lipoxygenase activity was found in the soluble fraction at 65% saturation. The per cent of activity from the crude extract recovered in the soluble fractions at each saturation level was: 35% saturation, 3%; 20% saturation, 74%; 0% saturation, 7%; insoluble residue resuspended, 0%. By elution from CM-cellulose at pH 4.8, the active portion was separated into two bands, each representing *ca* 50% of the total activity recovered. Upon concentration, each fraction developed a red color and showed strong peroxidase activity as well as lipoxygenase.

Lipoxygenase 1. Peroxidase did not bind to DEAE-cellulose at pH 7.8 so could be separated from lipoxygenase 1, which was weakly bound above pH 7. However, lipoxygenase 1 was not stable above pH 7, and two other activity bands (1_b and 1_c) trailed lipoxygenase 1_a from DEAE-cellulose, their proportions depending on the length of time lipoxygenase 1 was exposed to pH 7.8 prior to chromatography. (Lipoxygenase 1_a is used to designate that fraction of lipoxygenase 1 which, after exposure to pH above 7, eluted from DEAE-cellulose in the same position as lipoxygenase 1. Lipoxygenase 1_b and 1_c designate the other two bands eluted at pH 7.8.) As an example, when a preparation of lipoxygenase 1 was held at pH 7.8 for 3 days before fractionation, only 15% of the recovered activity was lipoxygenase 1_a (Table 1). Lipoxygenase 1_c was the only fraction which was stable above pH 7 at 2° (Table 1) or at −15°. In a purification of lipoxygenase in which the time to complete purification was minimized, 98% of lipoxygenase 1 was recovered as 1_a from DEAE-cellulose. It had a sp. act. of 4.3×10^{-5} kat/mg protein representing a 40 000-fold purification. This 1_a preparation retained activity for 1–2 months when stored at a pH lower than 7. Lipoxygenase 1 showed almost no tendency to separate into other forms when developed on DEAE-cellulose

Table 1. The integrity of lipoxygenase 1 was determined, after storage for 3 days at 2° in 0.019 M Pi buffer, pH 7.8, by development on DEAE-cellulose using a NaCl gradient, 0–50 mM, in Pi buffer, pH 7.8. The stability of the resulting fractions was measured after 2° storage for 18 days in Pi buffer, pH 7.8. Lipoxygenase was measured by polarography in 3 ml 1.2×10^{-4} M linoleic acid in 0.05 M Pi buffer, pH 6.8

Lipoxygenase fraction	% of total activity from DEAE-cellulose	Specific activity (kat $\times 10^{-6}$/mg protein) Day 1	Day 18	Day 18/Day 1
1_a	15	1.5	0.17	0.11
1_b	3	3.5	1.1	0.31
1_c	81	1.8	1.55	0.86

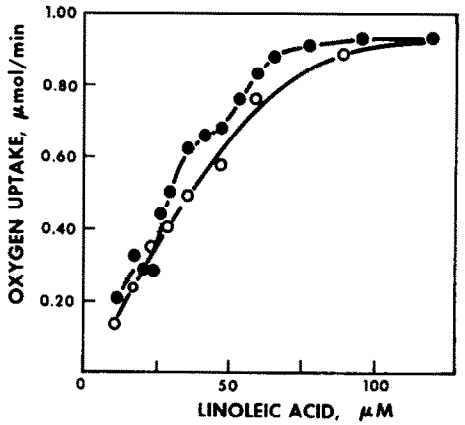

Fig. 1. Effect of linoleic acid concentration on the reaction rates of lipoxygenase 1 and 2 at pH 6.8. Reaction system: lipoxygenase 1—5 μg protein/3.0 ml, ○—○; lipoxygenase 2—19 μg protein/3.0 ml, ●—●. Lipoxygenase activity was measured by polarography.

below pH 7; a trace of lipoxygenase 1_b was obtained at pH 6.1 and at pH 6.8 but no lipoxygenase 1_c was detected.

Gel electrophoresis at pH 8.9 of preparations representing each band separated by passage of lipoxygenase 1 through DEAE-cellulose revealed that the enzyme(s) was not pure but that inactive protein bands were present in each of the preparations. Lipoxygenase 1_a was separated into two active bands at R_f 0.29 and 0.37. Lipoxygenase 1_b contained one very weak active band corresponding to a protein band at R_f 0.29, and lipoxygenase 1_c had a single major protein band at R_f 0.34 which corresponded to the only band with lipoxygenase activity. MW determinations indicated that lipoxygenase 1_a had two principal bands of equal intensity at 89000 and 86000 and a shoulder at 81000. Lipoxygenase 1_b had a MW of 83000, while lipoxygenase 1_c had a band corresponding to a MW of 83000 and a second band of slightly greater intensity at 79000. The absolute values for MW varied by 3–4000 between runs due to variability in conditions. However, the differences in MWs between fractions were consistent.

Lipoxygenase 2. Peroxidase and lipoxygenase 2 were separated by a combined pH and concentration gradient through CM-cellulose. Part (20%) of the recovered lipoxygenase was eluted near the front and the remainder formed a sharp band of activity trailed by inactive proteins. Kinetic analyses of the lipoxygenase 2 from the two active bands revealed no differences between them. The preparation of highest sp. act. obtained for lipoxygenase 2 (sp. act. 1.3×10^{-6} kat/mg protein representing 1200-fold purification) had a major protein band (R_f 0.17) and three minor bands in the electrophoretic pattern. The position of the major protein band corresponded to the lipoxygenase band. The MW of the major band was estimated at 84000 with two faint bands of 79000 and 77000.

Comparison of isoenzymes. The two isoenzymes possess both similarities and differences (Table 2). The lag period exhibited by lipoxygenase 2 was influenced by enzyme concentration, substrate concentration, or the presence of linoleate hydroperoxide in the initial reaction mixture [13]. In a plot of v_0 vs [S] a striking dissimilarity between lipoxygenase 1 and 2 was apparent (Fig. 1). Lipoxygenase 2 showed a pronounced irregularity at *ca* 20 μM linoleate not evident in the plot for lipoxygenase 1. A double-reciprocal plot relating initial velocity to substrate concentration for either lipoxygenase 1 or 2 did not produce a straight line but rather a sigmoidal curve for the isoenzyme.

When substrate stock solution (pH 10.5) was added to the reaction mixture (pH 6.8) at a final concentration well above the critical micelle concentration, followed by mixing for several min, a visible change occurred in the substrate. The initially clear solution gradually became cloudy. Concurrent with this change, the reactivity of the substrate also was altered (Fig. 2). The longer the substrate pre-mixed before enzyme was added, the lower was the

Table 2. A comparison of some characteristics of two wheat lipoxygenase isoenzymes

Subject of comparison	Lipoxygenase 1	Lipoxygenase 2
pH Optimum	Between pH 6 and pH 7; the maximum determined by substrate concentration [12]	
Stability when preincubated in the following compounds:		
(1) *p*-Chloromercuriphenylsulfonic acid (PCMS) (2.5 mg ml^{-1})	100% of original activity after 18 hr	50% of original activity after 10 min; no activity after 2 hr
(2) *N*-Ethylmaleimide (2.5 mg ml^{-1})	100% of original activity after 18 hr	Totally inactivated, but more slowly than by PCMS
(3) Dithiothreitol (5 mM)	50% of original activity after 2 hr	50% of original activity after 2 hr
(4) NaN_3 (5 mM)	0–50% of activity of control after 4 days	0–50% of activity of control after 4 days
Presence of lag period in reaction with linoleic acid	No measurable lag period	Initial lag period

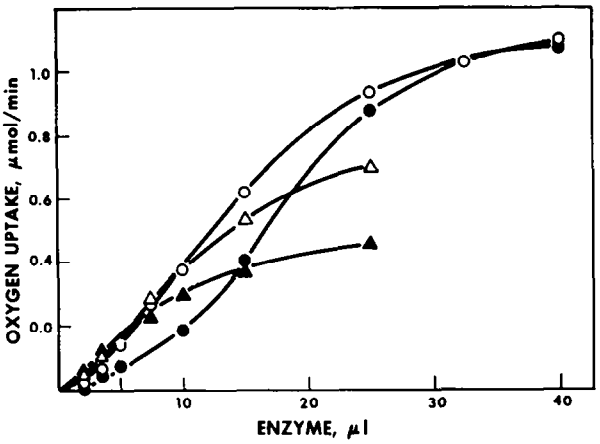

Fig. 2. Changes in the initial rate of oxidation of 200 μM linoleic acid caused by premixing in air in Pi buffer, pH 6.8, prior to introduction of lipoxygenase 1. Mixing time: O—O, 30 sec; ●—●, 5 min; △—△, 10 min; ▲—▲, 20 min. Lipoxygenase activity was measured by polarography.

maximum rate of reaction obtained with higher enzyme concentrations. No absorption at 234 nm, indicative of linoleate hydroperoxide formation, was found even after 20 min of mixing. This phenomenon did not substantially interfere with the studies of enzyme kinetics which were generally made during the first 30 sec of a reaction.

Characterization of the activator

Gel electrophoresis of the activator preparation collected from DEAE-cellulose yielded two protein bands: a sharp band with R_f 0.33, and a broader band R_f 0.12. Activation of the lipoxygenase reaction occurred with extracts of the two segments overlapping the R_f 0.33 position. Studies were done with the preparation eluted from the DEAE-cellulose. The activator was non-specific toward lipoxygenase and caused an increase in the level of activity with both lipoxygenase 1 and 2 as well as with a commercial preparation of soybean lipoxygenase measured at pH 6.8 in the standard Pi buffer. The MW of the activator is estimated at less than 20000 as it was only measured under conditions accurate for determinations over 20000, and it migrated beyond that range.

The activator varied in its effect upon reaction rate. In general, the increase in rate was not more than doubled under the conditions tested. Comparing curves of v_0 vs log [S] for lipoxygenase 2 with and without activator (Fig. 3), the activator essentially removed the perturbation in the rate curve for lipoxygenase 2 at about 20 μM (log 1.3) linoleate. The sigmoidal curve was markedly straightened in the presence of activator. Activation was not a linear function of the activator. The activation of lipoxygenase 1 diminished as the substrate concentration increased in the presence of a fixed amount of activator. The activator did not produce rate increases proportional to its concentration. At a given substrate concentration, the smaller the amount of activator present the greater the proportional increase in lipoxygenase activity over the control.

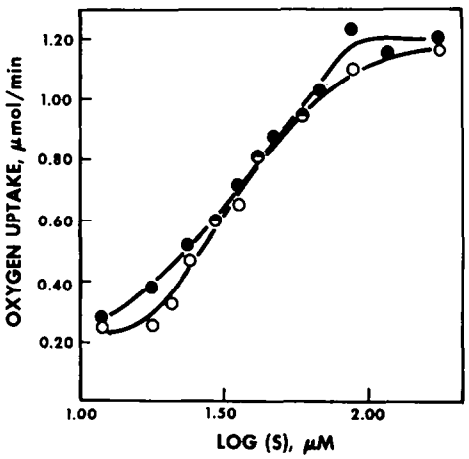

Fig. 3. Influence of activator on velocity of O_2 uptake by lipoxygenase 2 as substrate concentration (expressed as log [S]) was varied at pH 6.8. Reaction system: lipoxygenase 2—19 μg; activator—16 μg/3.0 ml. O—O, lipoxygenase 2; ●—●, lipoxygenase 2 plus activator. Lipoxygenase activity was measured by polarography.

DISCUSSION

Lipoxygenase activity in wheat mill fractions has been identified with 4 bands developed by disc electrophoresis [10, 11]. Two active fractions were separated from wheat flour [14]. An earlier report from this laboratory described 4 lipoxygenase fractions found in wheat germ [12]. The present account describes further purification with the conclusion that only two isoenzymes exist in wheat, but that one of them, lipoxygenase 1, is unstable in alkaline solution, and produced the multiple bands previously reported. Relative mobility of lipoxygenase bands in disc electrophoresis depended upon the pH of the sample, the pore size of the stacking and sample gels, and the wheat samples [10, 11]. Since the disc electrophoresis was carried out at pH 8.5, changes may have occurred in the isoenzymes. Two lipoxygenase fractions from flour were separated by stepwise elution at pH 5.9 and pH 7.5 from CM-Sephadex [14]. We do not know whether those preparations represent fractions corresponding to lipoxygenase 1 and 2 since different separation techniques were used, but *ca* four times as much activity was obtained by elution at pH 5.9 as at pH 7.5.

All of our isoenzyme preparations showed some inactive protein bands in electrophoresis. Since the major band in each case corresponded to the active enzyme, it was assumed that the major band in the SDS-gel electrophoresis also corresponded to the lipoxygenase fraction. However, the MW studies of the lipoxygenase 1 series revealed either two nearly equal bands or a band with a shoulder for each form of the isoenzyme. We could not distinguish between these bands on the basis of activity. The data suggests that small segments of the molecules of MW 89000 and 86000 were lost upon exposure to alkaline conditions. This explanation is supported by the time dependence of the changes in lipoxygenase 1. The MW of *ca* 84000–88000 for the isoenzymes fall half way between the 102000 for soybean [15] and 72000 for pea lipoxygenase [16]. Another possibility is that the double bands which appeared in the SDS-gel electrophoresis of lipoxygenase 1 represent constituents of a dimer.

Lipoxygenase 2 activity was destroyed in the presence of sulfhydryl reagents but lipoxygenase 1 was insensitive,

which suggests significant differences in the chemical groups governing their reactive sites. Gradual inactivation of the isoenzymes was observed over several days when exposed to azide. This observation would agree with the detection of iron in lipoxygenase [17, 18]. A constant and distinguishing feature between lipoxygenase 1 and 2 was the absence of lag phase with the former and its presence in catalysis by the latter [13]. The oxygen electrode had a response time of *ca* 5 sec so that lag periods shorter than that would not have been detected. Soybean lipoxygenase-1 exhibited a lag phase only above 2.5×10^{-5} M linoleate at pH 9.0 [19]. We found that lipoxygenase 2 exhibited a lag phase at the lowest substrate concentration tested, 10 μM, at pH 6.8. The sigmoidal curve for the double-reciprocal plot of v_0 vs [S] might be caused by the existence of some form of positive cooperativity. Smith and Lands [20] have already suggested that more than one binding site exists for soybean lipoxygenase.

The dissimilar forms of the initial rate curves for lipoxygenase 1 and 2 (Fig. 1) could be caused by a greater influence to reactivity with lipoxygenase 2 than with 1 by structural changes of linoleate in the region of the critical micelle concentration [21]. Indeed, the curve for lipoxygenase 2 has irregularities at about the same linoleate concentrations (20–40 μM) as Allen observed with soybean lipoxygenase-1. The demonstration that the maximum initial velocity of the isoenzymes was apparently dependent upon the structure of the substrate at high concentrations suggests that the efficiency of the reaction of enzyme with substrate decreases as micelle size increases.

The influence of the activator appeared to be associated with substrate concentration with both isoenzymes. The fact that the activator eliminated the perturbation in the initial rate curve for lipoxygenase 2 is suggestive that it may affect the critical micelle concentration of the substrate [19, 21]. Perhaps the activator, through ionic interaction, alters the substrate and indirectly influences the lipoxygenase activity. Where the activator is located in the intact tissue and whether it has a function related to lipoxygenase is not known. We suggest, based upon the diversity in their reaction characteristics, that two isoenzymes of lipoxygenase exist in wheat germ, as well as a molecule which interacts with the substrate to activate these isoenzymes.

EXPERIMENTAL

Wheat germ was defatted by extracting × 2 with Me_2CO at 2°. Linoleic acid was prepared and stored as described in ref. [13]. MW standards were: carboxypeptidase, 34 600; human serum transferrin, 75 000; polyovalbumin, 43 000, 86 000, 129 000 and 172 000.

Enzyme assays. Peroxidase activity was determined by a spectrophotometric method [22]. Lipoxygenase activity was determined with a Clark O_2 electrode, in a final vol. of 3 ml containing 1.2×10^{-4} M linoleic acid in 0.05 M Pi buffer, pH 6.8 unless otherwise stated. Enzyme was added to initiate the reaction. Protein was determined by the method of ref. [23].

Enzyme preparation. Defatted wheat germ was extracted for 1 hr in 5 parts (w/v) 0.18 M NaOAc, pH 4.8. This and all succeeding steps were performed at 2–5°. After centrifugation, the supernatant was brought to 65% satn with $(NH_4)_2SO_4$, centrifuged and the pellet partially dissolved in 35% satd $(NH_4)_2SO_4$ in NaOAc buffer. The lipoxygenase was dissolved from the pellet in 20% satd $(NH_4)_2SO_4$ in NaOAc buffer. The soln of enzyme in this and all following enzyme prepns was reduced in vol. by a simplified version of a pressure dialysis ultrafiltration method [24], namely, heavy-walled dialysis tubing was suspended inside an evacuated suction flask. After concn, several vols. of 0.07 M NaOAc buffer, pH 4.8, were passed through the sample to equilibrate it for ion exchange chromatography with CM-cellulose (column 1.2 × 40 cm). This was performed using a linear gradient developed with equal vols. of 0.07 and 0.77 M NaOAc buffer, pH 4.8. Fractions (8 ml × 25) were collected. Lipoxygenase was eluted as 2 bands at *ca* 0.3 M (lipoxygenase 1) and 0.4 M (lipoxygenase 2) NaOAc buffer. Peroxidase was present in both bands.

Lipoxygenase 1 was equilibrated with 0.019 M Pi buffer, pH 7.8 and then subjected to chromatography through DEAE-cellulose (column 0.8 × 45 cm) using a NaCl gradient (0–50 mM) in Pi buffer and 50 fractions (2 ml) were collected. Lipoxygenase activity was eluted at *ca* 10, 20 and 40 mM NaCl. Peroxidase was eluted at the front of the gradient. The pooled fractions from each lipoxygenase band (1_a, 1_b and 1_c) were concd and stored at −15°, although only 1_c was stable for more than a few weeks. Because of the instability, gel electrophoresis, SDS-gel electrophoresis and enzyme kinetics studies were conducted on freshly prepared fractions. Lipoxygenase 2 was further purified by ion exchange chromatography in a CM-cellulose column (1.2 × 45 cm) which employed combined pH gradient (pH 6.6–7.6), NaCl gradient (0–0.1 M NaCl) and Pi gradient. The gradients were established in a 3-chamber mixer containing 100 ml each of 33 mM Pi buffer, pH 6.6; 94 mM Pi buffer, pH 7.6; and 0.1 M NaCl in 94 mM Pi buffer, pH 7.6, in chambers 1–3, respectively. Fractions (3.7 ml × 80) were collected. Part (20%) of the lipoxygenase 2 was located in fractions 12 and 13, just after a large band of inactive protein eluted at the front (fractions 7–11). The remainder of lipoxygenase 2 was found in fractions 18–19, well separated from peroxidase which was detected in fractions 27 through 57. Fraction 18, of high specific activity, was used for further study. It was stable at −15°.

Gel electrophoresis, protein staining, and lipoxygenase detection. Precast 4% acrylamide gels (Bio-Rad Laboratories, Richmond, CA, U.S.A.) (0.55 × 10 cm) were equilibrated with pH 8.9 buffer composed of (0.188 M Tris–0.188 M glycine; pH 8.9)–0.188 M NaCl (96:4). This was the minimum salt concn required for lipoxygenase to enter the gel. The sample (5–80 μg protein for lipoxygenase activity, 8–30 μg protein for staining), mixed with 0.1% bromophenol blue and 5% sucrose, was introduced into the gel at 1 mA/tube and then a constant 13 V/tube was maintained for 2–2.5 hr in a compartment at 8°. Electrophoresis was stopped when marker dyes were approaching the ends of the gels. The gels were removed from the tubes and, immediately, either stained for protein or tested for lipoxygenase activity. The position of the tracking dye was marked with drafting ink [25]. To stain proteins, the gels were fixed for 18 hr at 4° in 15% TCA–25% *iso*PrOH [25], stained for 24–48 hr in 0.02% Coomassie Brilliant Blue in 12.5% TCA, destained and stored in 7.5% HOAc (changed once after 24 hr). Proteins were scanned at 550 nm. Lipoxygenase activity was detected by rinsing the gel surface with H_2O, then repeatedly applying substrate mixture ((36 mM linoleic acid stock containing 0.25% Tween 20)–(50 mM Pi buffer; pH 6.8)–glycerol) (2:1:9) to the gel at 10 min intervals for 1 hr. Excess substrate was removed by a glycerol rinse, the gel blotted dry, then the surface dusted with soluble starch powder and finally sprayed with freshly prepared satd KI soln. Yellow bands, indicating lipoxygenase activity, appeared within a few min and faded within 1 hr. Results were recorded on black and white film exposed through a Wratten No. 38A filter.

MW determination by SDS-gel electrophoresis. Precast acrylamide gels described above were equilibrated with pH 6.1 buffer composed of 0.205 M Tris, 0.205 M NaOAc, and 0.1% SDS. Protein samples were reduced [25] prior to electrophoresis. Reduced samples, mixed with 0.1% methyl green tracking dye and 5% sucrose, were introduced into the gels at not over 2 mA/tube, and then run at 9 V/tube for 2.5 hr. Proteins were stained and scanned as described above.

Lipoxygenase activator separation. A fraction which produced activation of lipoxygenase was separated from lipoxygenase 1 when developed on DEAE-cellulose at pH 7.8. A large protein band developed at the front and immediately behind and overlapping was the activator band. Lipoxygenase 1_a followed this band off the column. The activator was subjected to electrophoresis as described above, except that methyl green marker dye was used and the polarity was reversed. The fraction migrated toward the cathode. One gel was stained for proteins while 2 gels were cut into 3 mm segments, frozen and stored until analysed with lipoxygenase. Each segment was triturated in 3 ml Pi buffer, pH 6.9, in a conical centrifuge tube, the gel centrifuged, and the liquid tested for activity. Another portion of the activator was subjected to SDS-gel electrophoresis, as already described, to measure the MW.

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