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Lipoxygenase from Wheat. An Examination of Its Reaction Characteristics

Joan M. Wallace* and Edward L. Wheeler

Four lipoxygenase fractions were separated from wheat germ extract by DEAE-cellulose chromatography. The activity *vs.* pH curves for all four fractions varied as a function of linoleic acid substrate concentration. This behavior is interpreted as demonstrating a pH-dependent substrate inhibition of lipoxygenase. Evidence is presented that, in addition to linoleic acid hydroperoxide, an unidentified product was formed in the aerobic reaction of wheat germ lipoxygenase with linoleic acid.

Isoenzymes of lipoxygenase have been isolated from soybeans (Christopher *et al.*, 1970, 1972), peas (Eriksson and Svensson, 1970; Anstis and Friend, 1974), and alfalfa (Ben-Aziz *et al.*, 1971). The aerobic reaction of lipoxygenase with the *cis,cis*-1,4-pentadiene system of unsaturated fatty acids such as linoleic acid generally has been found to produce, as primary products, C-13 and/or C-9 conjugated diene hydroperoxides, depending on the source of the enzyme or on the isoenzymes under study. Graveland (1970a) demonstrated that unsaturated trihydroxy compounds were formed as secondary products of the aerobic reaction in wheat doughs but only if lipoxygenase was bound to glutenin during the reaction. Garssen *et al.* (1971, 1972) showed that a mixture of various dimers and unsaturated carbonyl monomers, as well as pentane, could be produced in an anaerobic reaction of soybean lipoxygenase in the presence of both linoleic acid and linoleic acid hydroperoxide. This paper describes preliminary results of the partial purification of four lipoxygenase fractions from wheat germ by DEAE-cellulose chromatography. All four fractions produced an unidentified early reaction product in addition to linoleic acid hydroperoxide during the aerobic reaction with linoleic acid.

EXPERIMENTAL SECTION

Materials. Linoleic acid (99+% pure) and Absorbosil 5 TLC plates were purchased from Applied Science Laboratories, Inc. Soybean lipoxygenase was obtained from Mann Research Laboratories and used without further purification. Sephadex G-200 was from Pharmacia Fine Chemicals, Inc., and DE-32 anion exchange cellulose from H. Reeve Angel, Inc.

Methods. To prepare linoleic acid substrate, 100 ml of sodium borate buffer (pH 9.0) containing 2.5×10^{-4} *M* EDTA was deaerated, after which 1 g of linoleic acid was added, under a stream of argon, to give a concentration of 36 m*M*. The mixture was sonicated under an argon atmosphere, transferred to storage vials, flushed three times with argon, and stored at −10°. For lipoxygenase activity measurements, this stock linoleate was diluted with phosphate buffer (pH 6.9) to give concentrations ranging from 0.06 to 3.6 m*M*. A solvent system of hexane–ethyl ether–glacial acetic acid (80:20:1) was used for tlc separations. Hydroperoxide concentrations were calculated from the absorbance at 234 nm, measured with a Perkin-Elmer M202 spectrophotometer, using a molar extinction coefficient of 25,000 l. mol^{-1} cm^{-1} (Johnston *et al.*, 1961).

Lipoxygenase activity was measured as the initial rate of O_2 uptake determined with a Clark oxygen electrode in a volume of 3 ml containing linoleic acid in concentrations up to 3.6 m*M*, and buffered with 0.025 *M* sodium phosphate (pH 6.9). One unit of activity is defined as the uptake of 1 μmol of O_2/min at 25°. The initial O_2 concentration in the reaction mixture was 0.24 m*M*, as determined by the method of Robinson and Cooper (1970). The method of Lowry *et al.* (1951) was used for protein determinations with bovine serum albumin for standardization.

Defatted wheat germ (50 g) was extracted for 1 hr at 2° with 5 vol of 0.12 *M* sodium phosphate buffer (pH 6.9) (all succeeding operations were carried out at 2°). Insoluble material was separated by centrifugation (31,000*g*, 25 min). The supernatant (210 ml) was adjusted to 35% saturation with $(NH_4)_2SO_4$, stirred 1 hr, and then centrifuged to remove the inactive precipitate. The resulting supernatant was brought to 55% saturation with $(NH_4)_2SO_4$ and the active precipitate was collected by centrifugation. This final supernatant contained negligible activity. The precipitate was dissolved in 0.12 *M* sodium phosphate buffer (pH 6.9) to a volume of 20.5 ml and dialyzed against the same buffer. This was accomplished by passing the buffer through the fibers of a Bio-Fiber 80 beaker (Bio-Rad Laboratories, Richmond, Calif.) at the rate of 16

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ml/min for 2 hr with intermittent agitation while the fibers were submerged in the enzyme solution within the beaker.

The dialyzed solubles were fractionated by filtration on a Sephadex G-200 column (4 cm × 85 cm) preequilibrated with 0.12 *M* sodium phosphate buffer. The lower portion of the column contained 325 ml net volume of 5-mm diameter glass beads mixed with Sephadex G-200 to extend the bed length and reduce compression of the gel (Crow and Rothfus, 1968). Lipoxygenase was eluted with the phosphate buffer. Enzyme activity was located in a single peak by spot testing (Wallace, 1972). The active samples were combined and concentrated to 12 ml with a Bio-Fiber 80 beaker whose cellulose acetate membrane is rated to retain 90–100% of substances with mol wt >100,000 and 65–90% with mol wt >60,000. The beaker, containing the solution to be concentrated, was placed in crushed ice, and vacuum applied to the hollow fibers which were in contact with the solution. A sevenfold concentration was achieved in 1 hr. This method for concentrating samples was preferred to lyophilization because, at best, only 50% enzyme activity could be obtained with the latter.

Further purification was accomplished with a DEAE-cellulose column (4 cm × 38 cm) previously equilibrated with 0.01 *M* sodium phosphate buffer (pH 6.9). The sample was placed on the column and elution begun immediately with a NaCl gradient in the same buffer. The salt gradient (Figure 1) was developed with a Buchler Instruments Varigrad mixer, using four chambers (400 ml each) containing 0.02, 0.06, 0.20, and 0.30 *M* NaCl. The active fractions were located by spot test and then quantitatively measured for activity with the oxygen electrode. The test tubes containing peaks DL_1, DL_2, and DL_3 were then pooled and concentrated. Peak DL_4 was not pooled to avoid possible contamination with the front of peak DL_2.

RESULTS AND DISCUSSION

Lipoxygenase Purification. The crude extract from wheat germ (Table I) had a specific activity 15- to 200-fold below that for other plant tissue such as pea (Eriksson and Svensson, 1970) or soybean (Stevens *et al.*, 1970). Fractionation by $(NH_4)_2SO_4$ resulted in a fourfold purification while retaining about 73% of the total enzyme. Passage through Sephadex G-200 separated the lipoxygenase from a large quantity of inactive protein which trailed the lipoxygenase or was not retained by the column. During concentration with the Bio-Fiber beaker the effluent from the Sephadex filtration lost protein other than lipoxygenase (Table I), even though the solution had already been exhaustively dialyzed with the same membrane prior to passage through the Sephadex column.

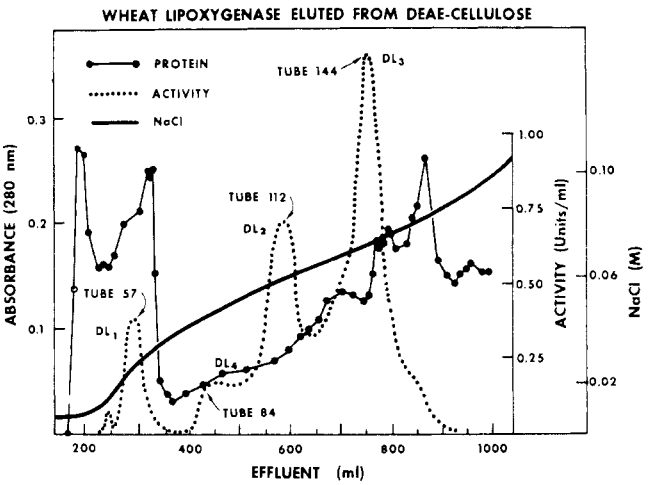

Figure 1. DEAE-cellulose chromatography of partially purified wheat germ lipoxygenase. The column was equilibrated with 0.1 *M* sodium phosphate buffer (pH 6.9), then the sample was applied, and gradient elution begun, as described in the text, starting with 0.02 *M* NaCl in phosphate buffer. Lipoxygenase activity was determined with 3.6 m*M* linoleic acid (pH 6.9).

Selective desorption from DEAE-cellulose resulted in the separation of four lipoxygenase fractions. Loss of protein again occurred when the DEAE-cellulose effluent fractions were reduced in volume (Table I). About 50% of the protein from DL_1 and DL_3 was lost during concentration of the fraction and, therefore, resulted in increased purity. A 15% overall recovery of lipoxygenase activity was realized from the initial wheat germ extract.

The elution profile from DEAE-cellulose (Figure 1) shows that although four peaks of lipoxygenase activity were identified, their contribution to the total protein of the samples was small as indicated by the absence of corresponding peaks in the protein profile. The highest value for specific activity, 6.4 for fraction DL_2 (Table I), represents 100-fold purification but is still very low when compared to the specific activity of lipoxygenase isolated from other plants. The four fractions which have been identified may be related to the four isoenzymes found by Guss *et al.* (1967) by gel electrophoresis of wheat mill fractions.

A tentative rough estimate of the minimum molecular

Table I. Lipoxygenase Activity during Isolation from Wheat Germ[a]

Purification step	Total protein, mg	Total act., units	Sp act., units/mg of protein	Degree of purification
Crude extract	13,340	860	0.064	1.0
35% $(NH_4)_2SO_4$ soluble	7,900	900	0.114	1.8
55% $(NH_4)_2SO_4$ ppt.	2,460	630	0.26	4.0
Sephadex effluent (dilute)	800	368	0.46	7.2
Sephadex effluent (concd)	670	370	0.55	8.6
DEAE-cellulose effluent				
DL_1 (dilute)	21	20	0.95	14.8
DL_1 (concd)	10.4	16	1.5	23.4
DL_2 (dilute)	8.0	52	6.5	102
DL_2 (concd)	7.4	48	6.4	100
DL_3 (dilute)	25	70	2.8	44
DL_3 (concd)	12	60	5.0	78
DL_4 (dilute)	0.66[b]	1.6[b]	2.4	38

[a] Recovery: DL_1 thru DL_3 (concd) + DL_4 (dilute) = 15%. Values given for 100 g of wheat germ. Linoleic acid (3.6 m*M*) used to determine activity. One unit of activity represents the initial rate of consumption of 1 μmol of O_2/min at 25°. [b] Measurement taken on 10 ml at peak.

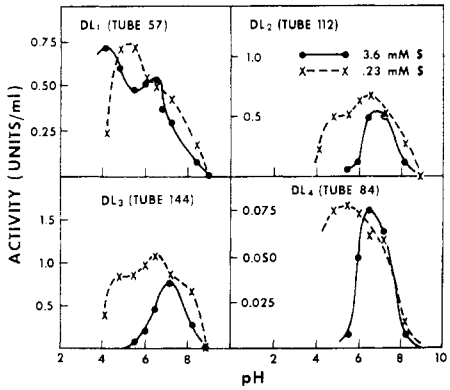

Figure 2. Lipoxygenase activity of four DEAE-cellulose fractions (indicated by tube number in Figure 1) as a function of pH at two concentrations of linoleic acid. Buffers used were 0.025 *M* acetate (pH 4.1–5.5), phosphate (pH 6.0–7.2), and borate (pH 8.2–8.9). Reaction was started by injection of enzyme solution into air-equilibrated buffer–substrate mixtures.

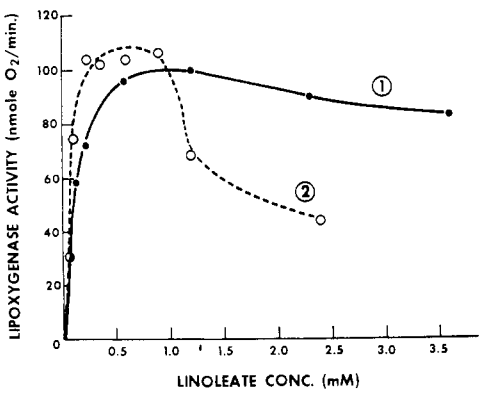

Figure 3. Activity of fraction DL_3 as a function of linoleate concentration at pH 6.9 using two batches of substrate. Curve 1: substrate ("older") prepared 2 weeks previously and vial opened under argon daily to remove portions. Vial flushed with argon and stored at −10° when not in use. Curve 2: "fresh" substrate prepared from linoleic acid on day used.

weight of wheat lipoxygenase can be made from the fact that little or no lipoxygenase was lost from the Sephadex or DEAE-cellulose effluents concentrated by suction through cellulose acetate membranes of a molecular exclusion limit of 90% for substances with >100,000 mol wt, and 65–90% exclusion limit for substances with >60,000 mol wt. This molecular weight would be in the range of soybean lipoxygenase (mol wt 102,000) (Theorell *et al.*, 1947) and pea lipoxygenase (mol wt 72,000) (Eriksson and Svenssen, 1970).

pH-Sensitive Substrate Inhibition. Aliquots from the peaks of the four lipoxygenase fractions were used to determine pH profiles at substrate concentrations of 3.6 and 0.23 m*M* (Figure 2). Generally, the relative lipoxygenase activity was greater at the lower substrate concentration, and the magnitude of the difference was a function of pH. Also, the pH optimum for each peak (with the exception of DL_1) was shifted to lower pH values at the lower substrate concentration. One explanation of these data is that lipoxygenase was inhibited by excess substrate, and the degree of inhibition was pH dependent. If this is so, the effect is probably not limited only to wheat lipoxygenase. Yamamoto *et al.* (1970) and Smith and Lands (1972) noted inhibition by substrate in reactions of soybean lipoxygenase. Ben-Aziz *et al.* (1970) found a shift in the relative activities of soybean lipoxygenase at pH 7.5 and 9.0, with a change in substrate concentration from 2.5 to 7.5 m*M*.

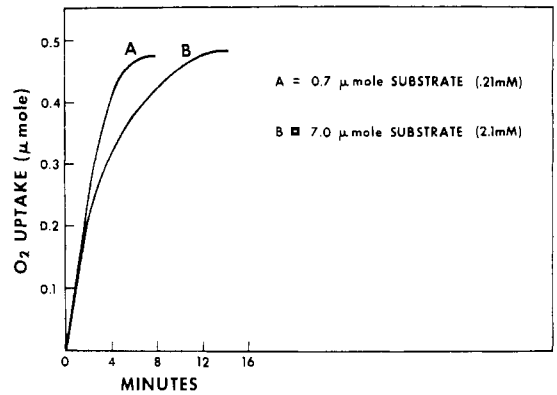

Figure 4. Lipoxygenase reactions at two concentrations of linoleate followed to the completion of O_2 uptake. Reaction started by injecting enzyme into 3 ml of 0.025 *M* phosphate buffer (pH 6.9), containing substrate and 0.7 μmol of O_2 (0.024 m*M*).

Effects of Autoxidation Products. In the course of our work we have sometimes obtained irreproducible lipoxygenase activity measurements, and the presence of traces of autoxidation products in the substrate may be responsible. Lipoxygenase activity can be modified by the condition of the substrate, even when the latter is only slightly oxidized (Figure 3). The initial rate of activity was greater with the "fresh" substrate at low concentrations, but at higher concentrations, the "fresh" substrate strongly inhibited the reaction, whereas the "older" substrate did not. The maximum rates of activity for the two substrates approached the same values but occurred at different concentrations. The uv difference spectrum of these substrates (with "fresh" substrate as reference and determined at a concentration of 0.12 m*M* in 80% ethanol) showed only 0.02–0.03 absorbance between 220 and 240 nm. No *peak* at 234 nm was found in either substrate, and tlc revealed traces of hydroperoxide and another product in only the "older" substrate. Smith and Lands (1972) suggested that hydroperoxide is necessary for initiation of the linoleic acid peroxidation reaction, and that substrate inhibition is caused by a competition between linoleate and hydroperoxide for a product binding site. If that were the only factor involved, then the results at lower substrate concentrations in Figure 3 suggest that more hydroperoxide might be present in the "fresh" than in the "older" substrate. However, this explanation does not seem to hold for the higher substrate concentrations where lower rates were observed with "fresh" linoleic acid. Actually, the ratio of linoleate:hydroperoxide would be constant throughout; thus the competition for binding sites on the enzyme would be independent of concentration. We can only suggest some as yet unidentified secondary product causes the shift in reaction rates.

Full Time Course of Reaction. In studies of the effect of substrate concentration on the full time course of reaction we found (Figure 4) that the reaction was retarded sooner after it was initiated at the higher than at the lower substrate concentration, yet the final extent of the reaction (O_2 consumed) was nearly the same at both substrate concentrations. Although not shown in the figure, oxygen uptake began again when more enzyme was added to curve B, suggesting total inactivation or inhibition of lipoxygenase in the presence of the higher substrate concentration as the reaction proceeded. Adding more enzyme did not reinitiate the reaction in curve A, in spite of data suggesting residual O_2 and linoleate. Since 0.5 μmol of O_2 had been used and 0.7 μmol of linoleate was initially present, there should have been 0.2 μmol of both O_2 and linoleate remaining. However, in this and similar experiments initially containing 0.7 μmol (0.24 m*M*) of substrate, the addition of more linoleic acid caused renewed

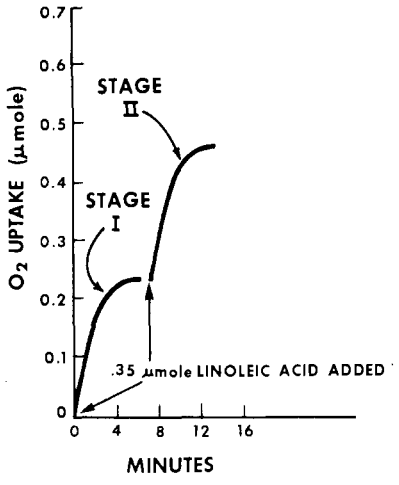

Figure 5. Oxygen uptake compared to hydroperoxide formed and to linoleic acid added in two steps. Reaction was started by injection of 0.35 μmol of linoleic acid (100 μl) into 2.8 ml of 0.025 *M* sodium phosphate buffer (pH 6.9), containing enzyme solution. After cessation of O_2 uptake, a 0.2-ml aliquot was withdrawn for hydroperoxide analysis and more linoleic acid injected to renew O_2 uptake. In stage I, 0.23 μmol of O_2 was consumed and 0.37 μmol of linoleic acid hydroperoxide was formed. In stage II, 0.25 μmol of O_2 was consumed and 0.38 μmol of linoleic acid hydroperoxide was formed.

O_2 uptake, indicating that the enzyme had not become inactivated, and also suggesting that the supply of original substrate had been exhausted.

In Figure 5, 0.35 μmol of linoleic acid was added to a mixture containing enzyme and 0.7 μmol of O_2 (stage I). The reaction stopped after only 0.23 μmol of O_2 had reacted, but a uv determination of hydroperoxide at that point indicated that 0.37 μmol of hydroperoxide had been formed, a ratio of O_2 consumed:hydroperoxide formed of only 0.62. The injection of more linoleic acid (stage II) yielded results similar to stage I. Our interpretation of these results is that in addition to hydroperoxide, another product which absorbs near 234 nm is formed by wheat germ lipoxygenase. It may be a primary product since, when "apparent" hydroperoxide formation and O_2 consumption were simultaneously determined at intervals during a reaction, the ratio of O_2 consumed to hydroperoxide formed was less than 1 at every stage (Table II). All four enzyme fractions from DEAE gave similar results, indicating that all fractions of the wheat germ enzyme reacted by the same course.

There was a difference in results between wheat germ and soybean lipoxygenase when the relative proportions of O_2 consumed and "apparent" hydroperoxide formed were compared after allowing the reaction to go to completion (Table III). If more substrate (2.1 μmol) than O_2 (0.7 μmol) was initially present, wheat lipoxygenase formed more hydroperoxide than would be expected from the initial amount of O_2. In every case with wheat lipoxygenase the O_2:hydroperoxide ratio was *ca.* 0.7.

For the soy lipoxygenase, on the other hand, with 0.24 μmol of substrate there was a nearly 1:1:1 correspondence among linoleic acid added, O_2 consumed, and hydroperoxide formed, as one would expect if hydroperoxide were the only product of the reaction. At 0.7 μmol of linoleic acid all of the available O_2 (0.7 μmol) was consumed, but only 0.54 μmol of hydroperoxide was apparently formed. In this sample, however, there was significant uv absorption at 285 nm in addition to the hydroperoxide absorption at 234 nm. This result can possibly be explained by the observation of Garssen *et al.* (1971), who found that soybean lipoxygenase anaerobically catalyzed the formation of diene conjugated carbonyl compounds (which absorb at 285 but not at 234 nm) from linoleic acid hydroperoxide. In the present case, this anaerobic reaction could have proceeded as the O_2 in the closed vessel became depleted, and the final concentration of hydroperoxide would have been lower than theoretical. In reactions with wheat lipoxygenase, we never observed any uv absorption at 285 nm, even in anaerobic reactions.

It is possible that dimeric products, such as those isolated by Garssen *et al.* (1972) from the anaerobic reaction of soybean lipoxygenase with linoleic acid and linoleic acid hydroperoxide, might be formed in the aerobic reaction of wheat germ lipoxygenase with linoleic acid, as the hydroperoxide and the dimers both absorb in the region of 234 nm and have similar molar absorptivity constants; but as demonstrated in Table II, they would have to be formed while O_2 is still in excess in the reaction. A diene conjugated hydroxy compound, such as described by Graveland (1970b), may be a possibility as its absorption characteristics fit with our findings. However, we have been unable to identify compounds such as these by tlc separation. Furthermore, Graveland's compounds were described as secondary products formed from lipoxygenase-mediated hydroperoxide by the action of other enzymes. It seems unlikely that a lipoperoxidase impurity could be present in the three lipoxygenase fractions, DL_1, DL_2, and DL_3, in such quantity to yield results such as shown in Table II during the early stage of the reaction. For these reasons, we believe some other product is formed.

Table II. Linoleic Acid Hydroperoxide Formed and O_2 Consumed at Intervals during Reaction with Wheat Germ Lipoxygenase[a]

Reaction time, min	Linoleic acid hydroperoxide formed,[b] μmol	O_2 consumed, μmol	Ratio O_2/ hydroperoxide
1	0.11	0.06	0.55
2	0.17	0.11	0.65
3	0.23	0.17	0.74
5	0.32	0.23	0.72
11	0.52	0.41	0.79

[a] Initial reaction conditions: 0.7 μmol of linoleic acid, 0.7 μmol of O_2, and 100 μl of DL_2 in 3.0-ml volume, pH 6.9, in a closed system. [b] Aliquot (0.20 ml) withdrawn and diluted with 80% ethanol, and linoleic acid hydroperoxide determined spectrophotometrically at 234 nm.

Table III. O_2 Consumed and Linoleic Acid Hydroperoxide Formed by Wheat Lipoxygenase and Soybean Lipoxygenase in Reactions Followed to Completion[a]

Enzyme source	Initial linoleic acid, μmol	O_2 consumed, μmol	Apparent L.A. hydroperoxide formed, μmol	O_2/linoleic acid hydroperoxide	Absorption (285 nm)
Wheat germ	2.1	0.55	0.80	0.69	0
	0.7	0.51	0.71	0.72	0
	0.35	0.21	0.30	0.70	0
Soybean	0.72	0.70	0.54	1.3	0.09
	0.24	0.26	0.23	1.1	0.01

[a] Initial reaction conditions: 0.7 μmol of O_2, sodium phosphate buffer (pH 6.9), 25°; reaction time, about 10 min.

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Specific Interactions of Linoleic Acid Hydroperoxides and Their Secondary Degraded Products with Enzyme Proteins

Setsuro Matsushita

The interaction of the enzymes, RNase, trypsin, pepsin and lipase with pure linoleic acid hydroperoxides and their secondary degraded products were examined in connection with the toxicity of oxidized lipids under mild conditions. The correlation of the inactivation of the enzymes to the incorporation of the autoxidized lipid products into the proteins and the consequent damage to the amino acid residues of the proteins was investigated. Polymerization of RNase by the autoxidized lipid products was determined.

Oxidation of unsaturated fatty acids leads to the formation of hydroperoxides and their secondary degraded products such as carbonyl compounds, acids, etc. Interactions of such compounds with proteins contribute to the destruction of their natural biological activities and hence cause toxicity to the biological systems. Several studies on the reactions of the autoxidized lipids with proteins have been reported by many authors (Desai and Tappel, 1963; Andrews *et al.*, 1965; Roubal and Tappel, 1966; Buttks, 1967; Little and O'Brien, 1968; Chio and Tappel, 1969). However, the reaction conditions which were used seemed to be too drastic to examine the toxic effects in biological systems, though they might be appropriate for determining the deterioration of food materials. The primary stable products of autoxidation, lipid hydroperoxides, were generally considered to be the cause of toxicity in lipid oxidation in biological systems, but recently emphasis has been placed on the toxicity of the secondary products (Schauenstein, 1967; Kaunitz and Slanetz, 1966; Kaunitz, 1967; Miura *et al.*, 1969; Yoshioka and Kaneda, 1972). Therefore, it becomes necessary to determine such toxic effects with purified hydroperoxides and secondary products more precisely and under mild conditions. In our earlier investigation (Matsushita *et al.*, 1970), detectable changes in enzyme activities were observed with partially purified linoleic acid hydroperoxides (LAHPO) at rather low concentrations. In the present paper, the interactions of the autoxidized products of linoleic acid, pure LAHPO, and secondary degraded products of LAHPO (SP) (mixture) against RNase, trypsin, and pepsin as model proteins are presented (Gamage and Matsushita, 1973; Gamage *et al.*, 1973). Effects on lipase activity are also discussed (Sohode *et al.*, 1974c). Some data of the effects of triglyceride hydroperoxides (TGHPO) on enzyme activities are also included. The reaction conditions were chosen to be as mild as possible in order to find the reaction mechanism of the autoxidized lipid products.

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MATERIALS

Linoleic acid (or linoleic acid-*1*-^{14}C) was autoxidized at 37° for 70 hr, and the autoxidized mixture was subjected to silica gel column chromatography (Gamage *et al.*, 1971) using 20% methanol-benzene as an immobile solvent and 2% methanol-benzene as a mobile solvent. The LAHPO fraction was further purified by preparative tlc using *n*-hexane-diethyl ether-acetic acid (60:40:1) as the solvent system. The LAHPO bands, as detected by uv light, were peeled off and recovered with methanol. The purity of LAHPO was confirmed by tlc, and the concentration of LAHPO was determined by uv absorption at 233 nm (O'Brien, 1969) and peroxide value (POV).

The SP fraction (ether eluate from the column) had a considerable POV. The SP fraction was further oxidized until no more POV could be detected. The SP was dissolved in methanol and the concentration was expressed as total carbonyls (Henick *et al.*, 1954).

TGHPO was fractionated from oxidized safflower oil by a similar procedure to that used for LAHPO (Sohode *et al.*, 1973, 1974a).

Bovine pancreatic RNase (5× crystallized), bovine pancreatic trypsin (2× crystallized), hog pepsin (2× crystallized), and porcine pancreatic lipase (Sigma type II crude) were purchased from Sigma Chemical Co.

RESULTS

Effects of LAHPO, SP, and TGHPO on Enzyme Activities. Enzymes were preincubated with LAHPO or SP at appropriate conditions (pH, concentration, tempera-