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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-

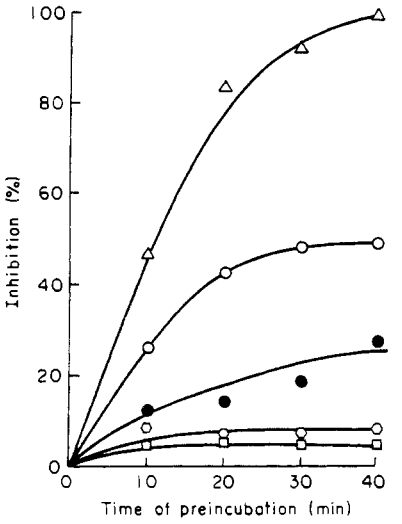

Figure 1. Effects of LAHPO and SP on RNase activity. RNase (0.01 mg) in 0.1 ml of solution was preincubated with 1.7 μmol of LAHPO or about 2 μmol of SP and 1 ml of 0.2 *M* sodium acetate buffer (pH 5.0) at 37°. At specific time intervals, the activity was determined. AsA, BHT, tryptophan, and uric acid were added at a final concentration of 1 μmol/ml before the addition of LAHPO or SP. The effects of the added compounds on the activity are expressed as per cent inhibition taking the original activity as 100%: (○) LAHPO; (□) LAHPO + BHT; (△) LAHPO + AsA; (○) LAHPO + tryptophan or uric acid (showed similar value); (●) SP.

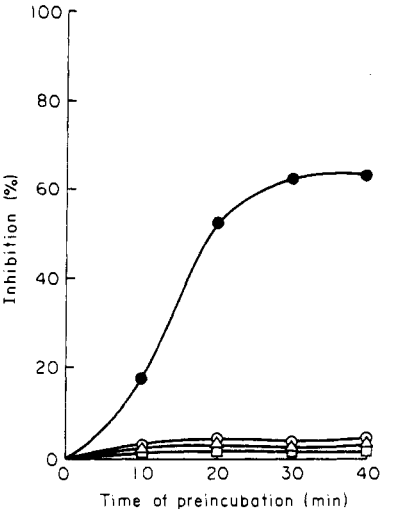

Figure 2. Effects of LAHPO and SP on trypsin activity. Trypsin (0.2 mg) in 0.1 ml of solution was preincubated with the same amount of LAHPO or SP as in Figure 1 and 1 ml of 0.2 *M* Tris buffer (pH 8.0) at 37°. The effects of AsA and BHT on the inactivation were determined as in the case of RNase. The symbolic representations and results are the same as in Figure 1.

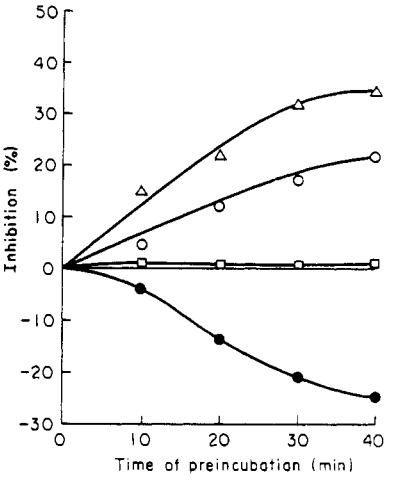

Figure 3. Effects of LAHPO and SP on pepsin activity. Pepsin (0.2 mg) in 0.1 ml of solution was preincubated with the same amount of LAHPO or SP and 1 ml of 0.2 *M* sodium acetate buffer (pH 5.0) at 37°. The effects of AsA and BHT on the inactivation were determined as in the case of RNase. The symbolic representations and results are the same as in Figure 1.

ture) as described in a previous paper (Matsushita *et al.*, 1970).

RNase was preincubated with LAHPO or SP. At specific intervals, inhibition of the enzyme activity was determined as per cent inhibition taking the original activity as 100% (Figure 1). The time course of inhibition increased up to 30 min of incubation beyond which it gradually approached a maximum. On the addition of ascorbic acid (AsA), the inhibition increased almost to completion, while butylated hydroxytoluene (BHT), tryptophan, and uric acid had a remarkable effect on the reduction of inhibition of the activity. SP inhibited the activity to a lesser extent than in the case of LAHPO. Higher concentrations of SP were considered incorrect as the higher concentrations produced intense turbidity. AsA or BHT had no appreciable influence on the activity of SP.

In the case of trypsin (Figure 2), LAHPO did not show any inhibitory effect on the activity. But SP showed a remarkable inhibition of the activity which was not affected by the addition of AsA or BHT.

LAHPO, under similar conditions as in the case of RNase, showed a similar pattern in the inhibition of pepsin activity but it was less remarkable (Figure 3). The effects of AsA and BHT on the action of LAHPO showed a similar pattern to RNase. SP under similar conditions had an activating effect on the activity of pepsin, which was not influenced by the addition of BHT, while AsA inhibited the activity.

TGHPO (emulsified with taurocholate or Tween 20) did not show any significant effect on the enzyme activities (Sohode *et al.*, 1974c) which were affected by LAHPO, while TGHPO showed similar bacteriostatic action on the growth of *Escherichia coli* as with LAHPO (Gamage *et al.*, 1971; Sohode *et al.*, 1974b).

Incorporation of LAHPO and SP into Proteins. Labeled LAHPO or SP was incubated with enzyme proteins. At certain intervals, the radioactivity incorporated into the enzyme protein was determined. In the case of RNase, the time course showed a relative increase in the incorporation. Table I shows the amount of incorporation after 40-min incubation. AsA enhanced the incorporation significantly. In the case of SP, the time course did not show any significant effect but the incorporated amount was more. AsA under similar conditions showed a promoting effect on the incorporation of SP into RNase while BHT had no effect.

LAHPO had no significant incorporation into trypsin. The presence of AsA in the reaction mixture showed an increase in the incorporation of LAHPO into trypsin while BHT had no effect. With SP, a remarkable incorporation was observed which was uninfluenced by the addition of AsA or BHT.

The incorporation of LAHPO or SP into pepsin, although it showed higher values, had no significant change with the time of incubation. The effects of AsA or BHT on the activity of LAHPO showed a similar pattern to that of ribonuclease (RNase). AsA had a considerable influence on the incorporation of SP into pepsin while BHT was ineffective.

Damage to the Amino Acids of the Enzyme Protein by the Interaction with LAHPO or SP. The enzyme proteins were incubated with LAHPO or SP and the unreacted samples or those bound to the protein nonspecifi-

Table I. Incorporation of LAHPO and SP into Three Enzyme Proteins[a]

Enzyme	LAHPO incorporated,% LAHPO only (nmol/mg of protein)	+BHT	+AsA	SP incorporated,% SP only (nmol/g of protein)	+BHT	+AsA
RNase	100 (32)	28	244	100 (88)	97	173
Trypsin	100 (16)	75	200	100 (142)	106	120
Pepsin	100 (43)	56	149	100 (115)	97	148

[a] LAHPO-*l*-^{14}C (2.5 μmol) in 0.05 ml of solution or labeled SP (about 2 μmol) in 0.01 ml of solution was added to 1 ml of enzyme solution [2 mg of protein in 0.2 *M* sodium acetate buffer (pH 5.0) for RNase and pepsin and 0.2 *M* Tris buffer (pH 8.0) for trypsin] and was incubated at 37° for 40 min. A certain volume of the reaction mixture was spotted on a tlc plate and developed with *n*-hexane–diethyl ether–acetic acid (60:40:1). The proteins left at the origin were scraped off and the radioactivity incorporated into the proteins was counted in a liquid scintillation counter. The effects of BHT and AsA on the incorporation were determined by adding the compounds to the reacting medium at a concentration of 1 μmol/ml before the addition of LAHPO or SP.

Table II. Damage to the Amino Acids of RNase, Trypsin, and Pepsin by the Interaction of LAHPO and SP[a]

Amino acid	% amino acid loss RNase +LAHPO	RNase +SP	Trypsin +LAHPO	Trypsin +SP	Pepsin +LAHPO	Pepsin +SP
Asp	0 (49)	0	0	0	0 (23)	25
Thr	0	0	14	17	0	0
Glu	10 (32)	0	0	14	13	0
Cys	40 (60)	0 (24)	0	50	0	19
Met	99	80	83	89	99	99
Leu	22	17	0	0	0	0
Tyr	62	17	0	20	0 (37)	0 (22)
Lys	51	50	0	0	0	22
His	54	49 •	12	42	0	0

[a] Enzyme solution (10 mg) in 1 ml of solution [0.2 *M* sodium acetate (pH 5.0) for RNase and pepsin and 0.2 *M* Tris buffer (pH 8.0) for trypsin] was incubated with 2.25 μmol of LAHPO or about 65 μmol of SP at 37° for 40 min. The protein solution was washed two times with chloroform and three times with diethyl ether and was hydrolyzed and chromatographically analyzed. On the determination of the effect of AsA, it was added to the reaction mixture at a final concentration of 1 μmol/ml before the addition of LAHPO or SP. The per cent amino acid loss was calculated using enzyme only as control; addition of AsA in parentheses.

cally were washed with chloroform and diethyl ether. The washed proteins were hydrolyzed, and the amino acid composition was determined. The damages to the various amino acids were elucidated by comparing with the respective amino acid of the native protein. Table II shows the damage to the amino acids of RNase by LAHPO or SP. Lysine, histidine, tyrosine, methionine, and cystine were the most labile amino acids to LAHPO attack while lysine, histidine, and methionine were the amino acids which showed susceptibility to SP interaction. On the addition of AsA to the reaction mixture containing LAHPO, aspartic acid and glutamic acid in addition to the amino acids damaged only by LAHPO were affected. AsA, when added to the reaction mixture containing SP, damaged cystine in addition to histidine and methionine.

Methionine was the only amino acid which showed lability to LAHPO in the case of trypsin, while methionine, cystine, and histidine were damaged by SP.

In the case of pepsin, only methionine was shown to be affected by LAHPO, while aspartic acid in addition to methionine were damaged by SP. Addition of AsA to the reaction mixture containing SP showed no significant change in the activity of SP with respect to the damage to the amino acids of pepsin. When AsA was added in the case of LAHPO, tyrosine and aspartic acid were damaged in addition to methionine.

Polymerization of Proteins by Radical and Nonradical Products of Oxidized Linoleic Acid. RNase when interacted with LAHPO was shown to contain at least two polymeric products (Figure 4), while the RNase allowed to react with SP (soluble fraction) was shown to contain one polymeric product (Figure 5). As shown in Figure 5, only a negligible amount of radioactivity is incorporated into RNase treated with SP. When AsA is present in the reaction mixture, about two times the amount of polymeric

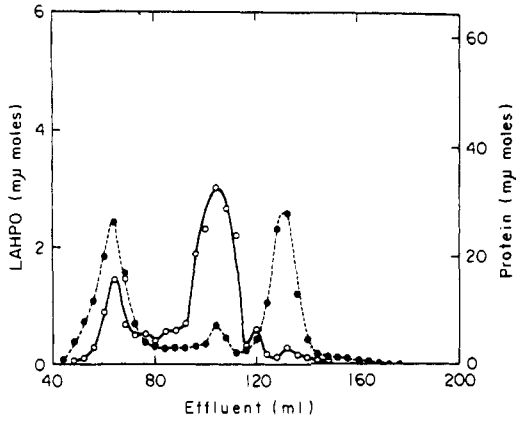

Figure 4. Sephadex gel filtration of the LAHPO-reacted RNase. RNase (10 mg) in 0.5 ml of 0.05 *M* sodium acetate buffer (pH 5.0) was incubated with 10 μmol of LAHPO-*l*-^{14}C at 37° for 4 hr. Free, bound, or associated oxidized products were eliminated from reacted proteins by ether extraction followed by solubilization in 3 *M* urea. The soluble fraction in 0.1 *M* Tris buffer (pH 8.0) was chromatographed with a Sephadex G-100 column using 0.1 *M* sodium acetate–0.2 *M* sodium chloride (pH 6.0) as the eluent (Chio and Tappel, 1969). Four-milliliter fractions were collected. An aliquot of the effluent fractions (0.5 ml) was taken for radioactive assay. The rest was subjected to protein determination. The RNase monomer was eluted at nearly the same position with that of unreacted LAHPO (130 ml). The enzyme activity was detected only during this part: (●) radioactivity; (○) protein concentration.

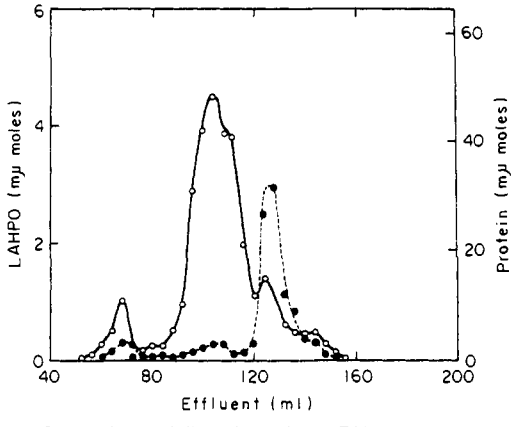

Figure 5. Sephadex gel filtration of the RNase reacting with SP. RNase (10 mg) in 0.5 ml of 0.05 *M* sodium acetate buffer (pH 5.0) was incubated with 5.4 μmol of labeled SP at 37° for 4 hr. The determinations and the presentations are as in Figure 4. The RNase monomer was eluted at nearly the same position with that of unreacted SP (130 ml).

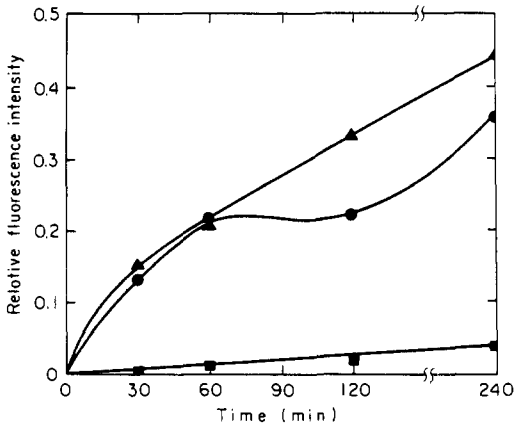

Figure 6. The relative production of fluorescence in RNase, trypsin, and pepsin by SP. RNase or pepsin (10 mg) in 0.5 ml of 0.05 *M* sodium acetate buffer (pH 5.0) or trypsin (10 mg) in 0.5 ml of 0.1 *M* Tris buffer (pH 8.0) was incubated with 5.4 μmol of SP at 37°: (●) RNase; (▲) trypsin; (■) pepsin.

products of RNase are formed by LAHPO in a 2-hr incubation compared to a 4-hr incubation without AsA (results not presented here; see Gamage *et al.*, 1973). Gel electrophoresis showed a broad band in the position corresponding to a RNase dimer. In contrast, BHT resulted in the reduction of polymeric products formed.

Incubating RNase with SP for 4 hr resulted in the formation of products which have fluorescence at 425 nm when excited at 355 nm (Figure 6). The reaction of trypsin was remarkable with respect to the production of fluorescent products.

Incubating pepsin with LAHPO or SP under the conditions used in the case of RNase did not produce polymeric products. The polymer formation of trypsin could not be detected under the conditions used due to the autolysis of trypsin.

Effects of LAHPO and TGHPO on Lipase Activity. Porcine pancreatic lipase was preincubated with LAHPO, TGHPO, or SP as shown in Table III. The extent of the inhibition of activity by TGHPO was less than by LAHPO. The inhibition could be recovered to some extent by the addition of BHT, AsA, and uric acid. SP did not show any inhibitory effect.

In addition, the availability of TGHPO as the substrate of lipase was tested. The liberation of LAHPO, due to hydrolysis of TGHPO by lipase, could be detected by tlc as shown in Figure 7.

Table III. Effects of LAHPO on Pancreatic Lipase[a]

Added compounds	Inhibition, %
LAHPO (10 μmol/ml)	88
LAHPO (2 μmol/ml)	57
LAHPO (2 μmol/ml) + BHT (1 μmol/ml)	41
LAHPO (2 μmol/ml) + AsA (1 μmol/ml)	44
LAHPO (2 μmol/ml) + uric acid (1 μmol/ml)	44
TGMHPO (10 μmol/ml)	17
TGMHPO (1 μmol/ml)	14
SP (2 mg/ml)	4

[a] Lipase (2 mg) in 0.2 ml of solution was preincubated with 0.05 ml of LAHPO, TGHPO, or SP emulsified with 5% gum arabic and 0.25 ml of 0.2 *M* acetate buffer (pH 5.0) at 37°. After 20 min, the activity was determined by the modified Doncombe method (Mori *et al.*, 1973).

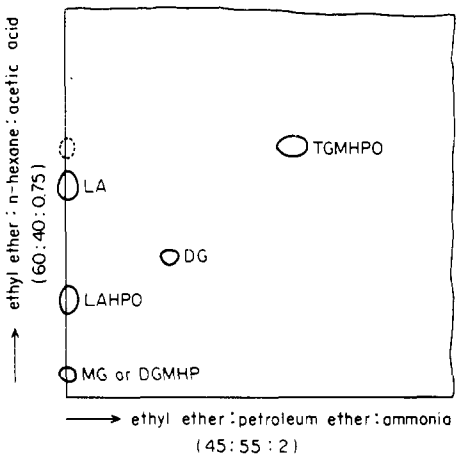

Figure 7. Two-dimensional tlc pattern of the digested products of TGMHPO with lipase. TGMHPO was digested with lipase at the same conditions as in the determination of lipase activity: LA, linoleic acid; LAHPO, linoleic acid hydroperoxide; TGMHPO, triglyceride monohydroperoxide; DG, diglyceride; MG, monoglyceride; DGMHPO, diglyceride monohydroperoxide.

DISCUSSION

LAHPO used throughout this study is pure and the methods used to determine the concentration may be considered to reveal almost exact values. The SP is a mixture of aldehydes, ketones, and acids of shorter chain length; under the conditions used in this study in determining the concentration of SP, the absolute values are not exact.

The inactivation of RNase by LAHPO (Figure 1) and the incorporation of LAHPO into RNase (Figure 4) showed similar tendencies. The inactivation seems to be due to incorporation. AsA enhanced the inactivation of the enzyme by LAHPO and the incorporation of LAHPO into the enzyme, while the antioxidants, BHT, tryptophan, (Marcuse, 1962) and uric acid (Matsushita *et al.*, 1963), reacted otherwise. This observation favors the assumption that LAHPO reacts on a radical mechanism. The amino acids shown to be damaged due to the reaction with LAHPO or the incorporation of LAHPO and hence the amino acids responsible for the inactivation of the enzyme were lysine, histidine, tyrosine, methionine, and cystine (Table I). Changes in the lysine and histidine residues which constitute the active site of RNase (Barnard and Stein, 1959; Smith, 1970) can inactivate the enzyme. The damage to the cystine residue can bring about conformational changes and inactivation of the enzyme (Setlow and Doyle, 1957; Cecil and Wake, 1962). The enhanced effect of AsA on the inactivation and incorporation may be attributed to the damage to aspartic acid and glutamic acid. Trypsin does not react much with LAHPO and hence a significant effect on the activity and considerable damage to amino acids cannot be expected. The tendency of pepsin to react with LAHPO is much less than that of RNase, and only methionine was shown to be affected.

Inactivation of RNase by SP was comparatively weaker than that by LAHPO. The weaker tendency to interact was further evidenced by the lesser number of amino acids which showed lability to SP than to LAHPO. SP showed a comparatively high tendency to react with trypsin as seen by the inactivation of trypsin and the incorporation of SP into trypsin. Consequently, more amino acids (histidine and cystine) were damaged by SP than LAHPO. The antioxidants or the prooxidants showed no effect on the activity of SP. In the case of pepsin, SP showed an activating effect on the activity while the presence of AsA inhibited the activity. Lipase was also affected by LAHPO, but TGHPO was less effective, and TGHPO could be digested by the enzyme. This fact seems to be interesting from the standpoint of food digestion. The fact that TGHPO had no effect on the enzymes used in the experiment may be explained by structural difficulty in attaching to the enzyme reactive site.

The radical intermediates and the nonradical products of peroxidized lipids are both effective in polymerizing RNase. The radicals formed by the fission of LAHPO may initiate a chain reaction resulting in the formation of protein radicals. These protein radicals react together to form polymeric products. This conclusion is in agreement with the observation of Roubal (1966), who suggested that the radical intermediates initiate the production of protein radicals which in turn polymerize as $P-(P)_n-P\cdot$. This mechanism is further strengthened by the effects of AsA and BHT on the polymer formation of RNase by LAHPO. The pronounced effect of AsA on the polymer formation of RNase by LAHPO suggests that AsA has increased the rate of the production of alkoxy or peroxy radicals. These radicals react with the proteins, affecting the formation of the protein radicals, which are also being degraded to SP. The antioxidative behavior of BHT further supports the radical mechanism of LAHPO.

The reaction of SP is time dependent and SP did not produce polymers within a short incubation period but showed a relatively high fluorescence in contrast to LAHPO. LAHPO did not produce the fluorescence at 425 nm, which is due to a carbonylamine condensation reaction resulting in the formation of a conjugated chromophoric Schiff's base system (Chio and Tappel, 1969).

From the differences observed in the incorporation, inactivation, and relative damage to the amino acid residues with respect to LAHPO and SP, it was deduced that different amino acid residues are able to be attached by LAHPO or SP. Among the four enzymes under consideration, the structural conformations are different. This results in differences of the surface exposed groups susceptible to attachment by LAHPO or SP, and such exposed groups may be influenced by pH. For instance, at pH 8.0 amino groups of protein would be more unprotonated and better nucleophiles than at pH 5.0. The occurrence of a conformational change of protein structure can also be expected from a change in pH. Also, pH may affect the formation of reactive groups, for instance, the differences of radical formation by the decomposition of LAHPO at pH 5.0 and 8.0.

The specific interactions are very complicated, and it can only be said that specific interactions of oxidized products of linoleic acid and proteins are caused by several factors, pH, specific structure of proteins, etc.

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Involvement of Cytochrome P-450 in the Intracellular Formation of Lipid Peroxides

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Organic hydroperoxides greatly increase the effectiveness of hemoproteins in catalyzing lipid peroxidation. Using this technique it has been shown that the heat-labile catalyst responsible for liver microsomal lipid peroxidation was cytochrome P-450. Evidence for this included inhibitor studies and reconstitution studies. Cytochrome P-450 had similar kinetics and turnover number to plant lipoxygenases and was more effective than other hemoproteins and hematin. A mechanism is proposed for lipid peroxidation in which the catalyst acts as a peroxidase with lipid as a hydrogen donor. A similar mechanism involving other hemoproteins is also presumably involved in the oxidative deterioration of lipid containing foods.

Polyunsaturated fatty acids, a major component of many vegetable oils, undergo autoxidation at ambient temperatures to yield hydroperoxides and a wide range of secondary degradation products associated with subsequent rancidity. Thus, high peroxide values occur after prolonged storage, exposure to sunlight, increased temperatures, and contact with iron or copper as in cooking vessels (Frazer, 1962). Dietary fatty acid hydroperoxides can be toxic to the gastrointestinal tract and can be carcinogenic (Cutler and Hayward, 1974; Cutler and Schneider, 1973). The identification of the catalysts in animal tissues which are responsible for the oxidation of unsaturated fats

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