Factors Affecting Product Specificity of Peanut Lipoxygenase[1]

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ABSTRACT

Product specificity of peanut lipoxygenase with linoleic acid as the substrate was determined under different conditions. Extraction solvent, extraction temperature, and reaction temperature only slightly affected the isomeric hydroperoxide ratio. A more pronounced effect was reflected in the total amount of hydroperoxide produced and extracted. $CHCl_3$:CH_3OH was the most thorough solvent, and extraction temperature of 25 C increased the total amount of hydroperoxides recovered. Lower reaction temperatures produced greater quantities of total hydroperoxides. Oxygen tension and pH value markedly affected changes in the hydroperoxide isomeric ratio and the total amount of hydroperoxides produced. Isomerization of the isolated hydroperoxides occurred very rapidly at 25 C, and a linear isomerization rate was observed at 4 C. At -20 C isolated hydroperoxides isomerized only slightly. Concentration of isolated hydroperoxides affected isomerization of the isomeric hydroperoxides; storing hydroperoxides in dilute solutions was most effective in preventing isomerization.

INTRODUCTION

The degradation of unsaturated lipids, enzymatic and nonenzymatic, proceeds by the production of isomeric hydroperoxides (1-4). Hydroperoxides are further decomposed or react with other components of the reaction mixture to form aldehydes, hydrocarbons, oxodiene compounds, and dimers (5). The exact physiological function of lipoxygenase in plants is unknown, but its apparent role in the production of desirable and undesirable volatile components in edible products is partially responsible for the concentrated efforts to understand lipid peroxidation (6,7).

Lipoxygenase occurs in many plants and can be generally identified by positional isomeric specificity, pH optimum, and other criteria. For example, the 13-hydroperoxide positional isomer is the predominant isomer produced from linoleic acid by soybean lipoxygenase at pH 9 and by peanut lipoxygeanse at pH 6.2 (4,8). Corn and potato lipoxygenases produce primarily the 9-hydroperoxide isomer from linoleic acid (8,9). Positional isomeric ratios produced by lipoxygenases with linoleic acid as substrate have been extensively studied (9-12). Reported isomeric ratio values (13:9 hydroperoxide) produced by soybean lipoxygenase vary from 100:0 (13) to 50:50 (10).

Positional and geometrical hydroperoxide isomers from a peanut lipoxygenase-linoleic acid reaction mixture have been isolated and identified in our laboratory (4). This study was initiated to determine the effect of different parameters on the isomeric ratio of lipoxygenase systems and to standardize the conditions for production, isolation, purification, and subsequent handling of hydroperoxides produced by lipoxygenase peroxidation of unsaturated fatty acids containing a *cis,cis* 1,4-pentadiene structure.

EXPERIMENTAL PROCEDURES

Hydroperoxide Formation

Standard reaction conditions were used to produce isomeric hydroperoxides by incubating peanut lipoxygenase with linoleic acid (high purity grade III from Sigma Chemical Co., St. Louis, MO) as described (4). Reaction mixtures were incubated at pH 6.2 or 8.3 and at 0 C, 22 C, and 37 C to determine effects of temperature and pH on isomeric ratios. Aerobic reaction conditions were obtained by saturating the system with oxygen during the entire reaction period. Oxygen-limiting conditions were accomplished by closing off the reaction flask after enzyme addition. All reactions continued for 30 min unless otherwise specified. For each reaction mixture, the conjugated diene content was determined spectrometrically at 234 nm in absolute

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TABLE I

Effect of Extraction Solvents, Extraction Temperature, Reaction Temperature, Oxygen Tension, and pH on Product Specificity of Peanut Lipoxygenase[a]

	% 13-LOOH *cis-trans*	% 13-LOOH *trans-trans*	% 9-LOOH[b]	Mg LOOH[c]
Extraction with $CHCl_3$:CH_3OH	70.4 ± 5.0	2.3 ± 0.6	27.3 ± 4.4	42.8 ± 9.0
Extraction with Ether	72.8 ± 2.9	1.9 ± 0.2	25.7 ± 2.8	33.6 ± 5.7
Extraction with Ether-Pet. Ether	72.8 ± 5.4	2.1 ± 1.1	25.1 ± 5.4	27.7 ± 4.7
Extraction at 4 C	77.4 ± 1.1	1.9 ± 0.6	20.7 ± 0.8	65.8 ± 8.8
Extraction at 25 C	74.6 ± 0.6	1.5 ± 0.3	23.9 ± 0.4	79.4 ± 7.3
Reaction at 0 C	82.9 ± 1.7	1.1 ± 0.5	16.0 ± 1.8	87.1 ± 4.0
Reaction at 225 C	80.7 ± 1.3	1.6 ± 0.6	17.7 ± 0.8	83.2 ± 5.6
Reaction at 37 C	77.8 ± 3.4	2.7 ± 0.5	19.5 ± 3.0	52.4 ± 6.1
pH 6.2	74.9 ± 1.5	2.6 ± 0.5	22.5 ± 1.2	41.8 ± 9.8
pH 8.3	42.7 ± 0.5	7.0 ± 1.3	50.3 ± 1.3	18.9 ± 1.5
Oxygen Saturated	74.0 ± 1.5	2.6 ± 0.5	22.5 ± 1.2	41.8 ± 9.8
Oxygen Limited	45.2 ± 3.6	10.9 ± 3.6	43.9 ± 5.8	7.7 ± 2.2

[a]Average of three replications ± SD.
[b]A mixture of *trans-trans* and *cis-trans*.
[c]Amount in the ether-hexane fraction.

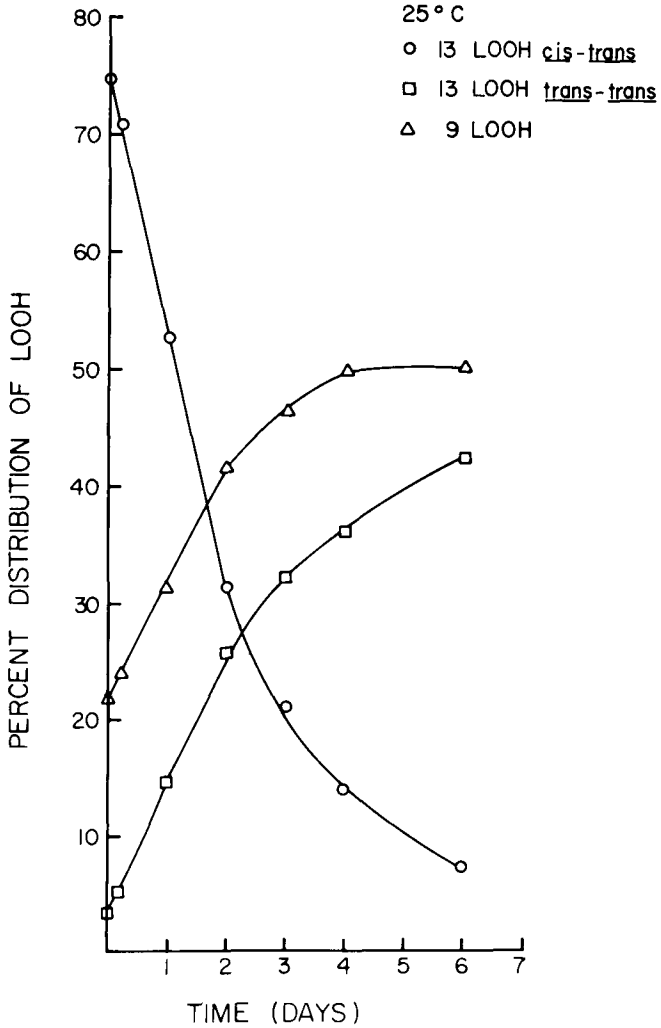

FIG. 1. Isomerization of hydroperoxides (5 mg/ml in hexane) stored at 25 C. 13-LOOH *cis,trans* (13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid); 13-LOOH *trans,trans* (13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoic acid); 9-LOOH (a mixture of 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoic acid and its corresponding *cis*-10,*trans*-12 isomer).

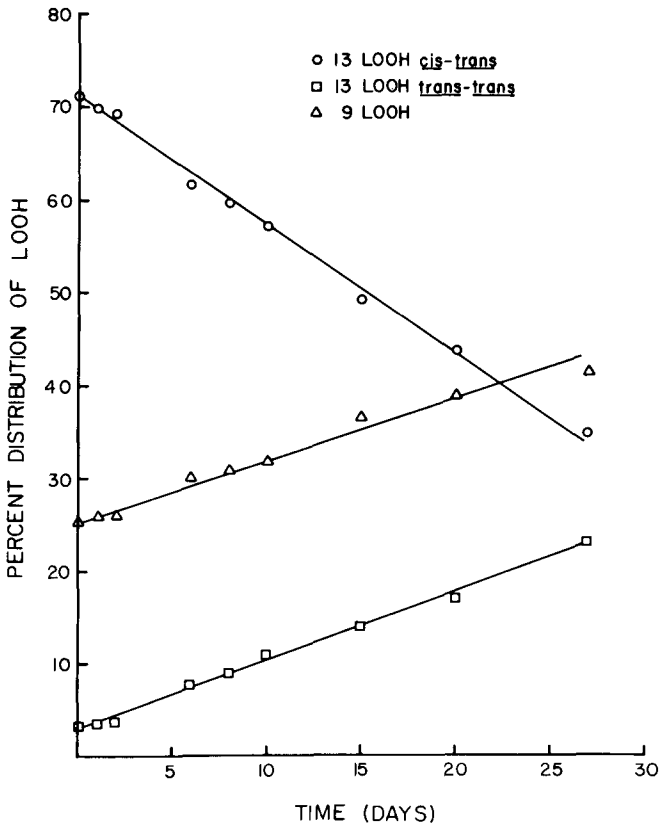

FIG. 2. Isomerization of isomeric hydroperoxides (5 mg/ml in hexane) stored at 4 C. See legend of Figure 1 for explanation of abbreviations.

ethanol prior to enzyme addition and after termination of the reaction. Total conjugated diene concentration produced in the reaction mixture was calculated using a molar extinction coefficient of 25,000 $\ell.mol^{-1}$, cm^{-1} at 234 nm (14).

Extraction and Methylation

Three solvents $CHC\ell_3$:CH_3OH (2:1, v/v), diethyl ether-petroleum ether (bp 60-110 C) (1:1, v/v), and diethyl ether were used independently to extract hydroperoxides from reaction mixtures after HCl was added to reduce the pH to ca. 3. Reaction mixtures (190 ml) were extracted three times with 200 ml of the respective solvent. Extracts were combined, washed three times with distilled H_2O, reduced in volume, and the hydroperoxides were methylated with diazomethane (16). Hydroperoxide methyl esters were purified by silica gel column chromatography (4) and the ether-hexane (1:1, v/v) fractions were used for further analysis.

High Performance Liquid Chromatography (HPLC)

Hydroperoxide methyl esters were separated by HPLC on a 4.6 x 500 cm Partisil 10 column using a Varian Model 8500 liquid chromatograph. Hydroperoxides were detected by monitoring the effluent at 234 nm with a Varian Varichrom detector equipped with 8 μl flow cells. Hexane was used as the eluting solvent with absolute ethanol as the polar modifier. Solvent flow rate was 4 ml/min, and the polar modifier solvent percentage varied from 0.26% to 0.4%. Isomeric ratios were determined by integrating the 234 nm absorption peaks with an Autolab System I Computing Integrator. Positional and geometrical isomers eluted by this system were identified by mass spectrometry and infrared spectrometry (4).

RESULTS AND DISCUSSION

For determination of product specificity of lipoxygenase reactions, analytical procedures must be controlled to minimize positional and geometrical isomerization. Table I shows the effect of solvent on isomeric ratios and extractability of reaction products from a peanut lipoxygenase-linoleic acid reaction. The extraction solvent used for product isolation had little effect on the ratio of isomeric hydroperoxides. Significant differences were observed in the total amount of conjugated diene products extracted by different solvents (Table I). Extractability increased with increasing solvent polarity, and a slight increase in the percentage of the positional isomer extracted was observed with $CHC\ell_3$:CH_3OH. Clearly the most polar solvent ($CHC\ell_3$:CH_3OH) produced the most thorough extraction of the conjugated diene reaction products.

According to infrared absorption studies, the more labile *cis,trans* isomers of hydroperoxides isomerize to the *trans,-trans* configuration with increasing temperature. However, the relationship of solvent-extraction temperatures to isomeric ratios and extractability of lipoxygenase reaction products has not been studied. Table I shows effects of extraction temperature on the isomeric ratio of hydroper-

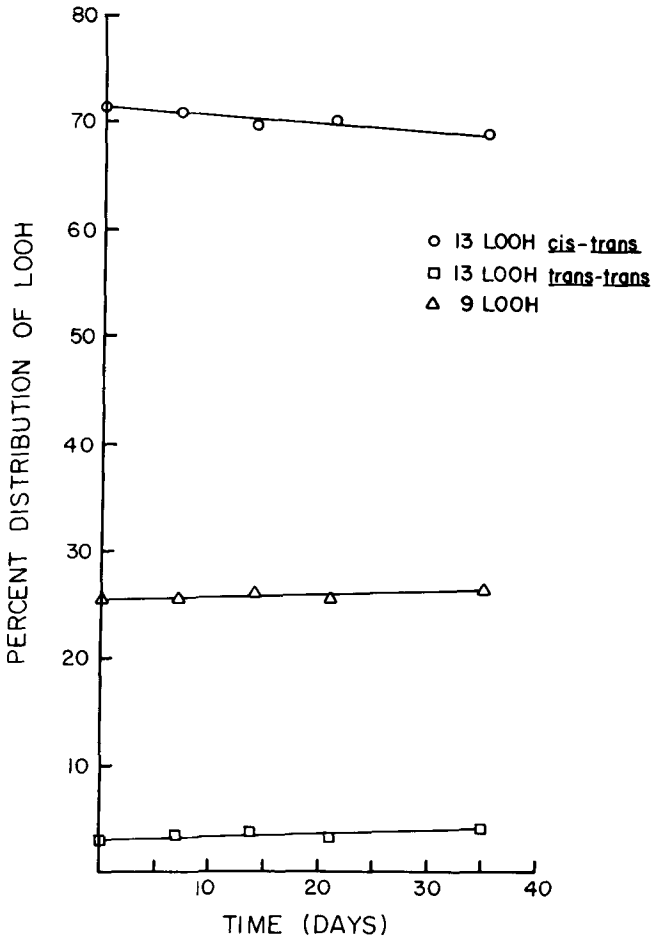

FIG. 3. Isomerization of isomeric hydroperoxides (5 mg/ml in hexane) stored at -20 C. See legend of Figure 1 for explanation of abbreviations.

oxides produced in the reaction. Higher extraction temperatures (25 C) gave an increased percentage of the 9-hydroperoxide (9-LOOH), and total extractability of conjugated diene reaction products improved. Extraction temperature had no pronounced effect on the isomeric ratio. However, some researchers include low-temperature extraction as an integral part of their methodology to prevent isomerizations during isolation and subsequent isomeric ratio determination by HPLC (3). Extractability and convenience make extraction at 25 C desirable.

Hydroperoxide isomerization can occur at any point during the work-up procedure. Adequate comparisons of different lipoxygenase reaction temperatures at a particular pH with regard to hydroperoxide isomerization have not been reported. Table I shows the effect of reaction temperature on positional and geometrical isomer ratios of hydroperoxides and on total amount of conjugated diene produced. Lower reaction temperatures (0 C) slightly favored the production of 13-LOOH *cis,trans* isomer. Amounts of 13-LOOH *cis,trans* isomer produced at three incubation temperatures did not differ as much as might have been expected since low reaction temperature (0 C) either enhances 13-LOOH *cis,trans* production or prevents isomerization. Increased amounts of total conjugated diene products were isolated as reaction temperatures decreased, and isomeric ratios were not greatly influenced by incubation temperature.

Oxygen is required for the conversion of unsaturated fatty acids to hydroperoxides by lipoxygenases, but the reaction proceeds under reduced oxygen tension. Table I shows an effect of oxygen on the production of isomeric

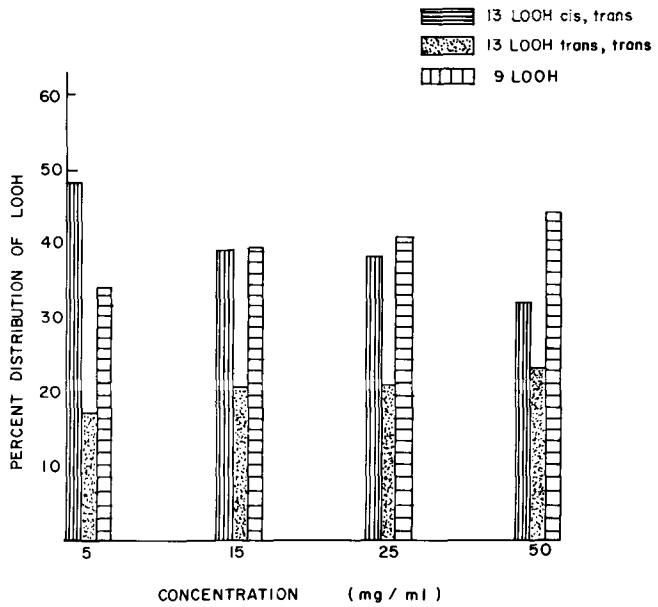

FIG. 4. Effect of concentration on the isomerization of isomeric hydroperoxides at 25 C (24 hr). See legend of Figure 1 for explanation of abbreviations.

hydroperoxides under saturated and reduced oxygen tensions. With oxygen saturation 13-LOOH isomeric species dominated; whereas reduced oxygen tension gave a ratio of about 1:1 for the positional isomeric compounds. Total amounts of conjugated diene produced with limited oxygen were reduced approximately one-half the amounts produced with a saturated oxygen environment. These results agree with isomeric ratios of positionsal isomers in soybeans reported by Leu (17) for reactions at pH 9 and 6 with 1% oxygen at 25 C. Leu and Eriksson (12), however, found that oxygen tension had essentially no effect on the isomeric ratio of hydroperoxides produced by pea lipoxygenase from linoleic acid. DeGroot et al. (18) proposed that in soybeans under anaerobic conditions an enzyme-ferric complex exists, and the free-radical enzyme complex was responsible for lipoxygenase activity under the conditions of anaerobiosis. However, the data they presented were not conclusive. It thus appears that the lipoxygenases from different sources vary in behavior with regard to products produced under reduced oxygen tension and with the specific analytical procedure applied. Other mechanistic factors besides reduced oxygen tension could have been operating to produce the 1:1 positional isomeric ratio observed in the peanut-lipoxygenase linoleic reaction.

Product specificity of peanut lipoxygenase was altered significantly by changing the pH of the reaction mixture. Table I shows pH effects with regard to product specificity of peanut lipoxygenase when reacted with linoleic acid. Predominance of 13-LOOH occurred at pH 6.2, and positional isomers approached a 1:1 ratio at pH 8.3. Total conjugated diene production decreased significantly when the reaction mixture was incubated at pH 8.3. Our results are in agreement with those of Christopher and Axelrod (10) who reported that at a neutral pH soybeans produced a 1:1 ratio of positional isomers; however, the authors stated that differences in isomeric ratio cannot be explained entirely on the basis of pH. Enzyme-reaction mixtures at pH 8.3 and pH 6.2 were incubated and analyzed under the same conditions in this study. Therefore, observed differences in isomeric ratios could not be attributed to autoxidation per se. Isomerizations may be primarily responsible for the observed 1:1 ratio of positional isomers at pH 8.3.

Conjugated diene hydroperoxides undergo chemical

isomerizations depending on environmental conditions and storage time prior to analysis. Occurrence of chemical artifacts has made determinations of product specificity for lipoxygenases very difficult. Figure 1 shows the chemical isomerizations which occurred with peanut-lipoxygenase-produced hydroperoxides that were held at 25 C for as long as 6 days. The 13-LOOH *cis,trans* compound isomerized rapidly to the more stable geometrical configuration at 25 C, and ca. 2¼ days were required for the 13-LOOH geometrical isomers to reach a 1:1 ratio. The 13-*trans,trans* isomer and 9-LOOH positional isomer increased at about the same rate, thereby indicating positional migration could have occurred simultaneously with geometrical isomerizations. However, other undetected intermediates could also have been involved in the isomerization mechanism. Previously, Chan et al. (3) reported thermal chemical isomerizations of soybean hydroperoxides at 42 C when incubated for 15 and 40 hr. Ratios (9 to 13) changed from 5:95 to 15:85 at 15 hr and 41:59 at 40 hr. Our data show (Fig. 1) that the 13-LOOH percentage decreased from 75 to 55 at room temperature in 24 hr. Isomerizations occurred very rapidly and without increasing the storage temperautre.

Figure 2 shows the results of storing isolated isomeric hydroperoxides at 4 C at a concentration of 5 mg/ml in hexane. Isomerizations were slower, but occurred at a linear rate for 25 days. The 13-*trans,trans* and the 9 positional hydroperoxides increased approximately at about equal rates indicating positional migration as well as geometrical isomerization. Approximately 22 days were required for the 13-LOOH isomeric hydroperoxides to reach a 1:1 ratio at 4 C as compared to 2¼ days at 25 C. Therefore, increased stability of isolated isomeric hydroperoxides was achieved at 4 C. However, this temperature was not adequate for prolonged storage.

Storage of isolated isomeric hydroperoxides at -20 C greatly improved the stability of hydroperoxides as shown in Figure 3. Over a 35 day period, only a 3% decrease in the 13-LOOH *cis,trans* hydroperoxide was observed at this temperature. The 13-LOOH *trans,trans* isomer and the 9-LOOH positional isomer increased at equal rates. At all three temperatures, the 13-LOOH *trans,trans* isomer and the 9-LOOH positional isomer increased at approximately the same rate although the ratio was different for each temperature. Apparently isolated isomeric hydroperoxides would be relatively stable during prolonged storage at -20 C.

The concentration of isomer hydroperoxides can also affect the degree of isomerization of samples for further analysis. Figure 4 shows changes occurring due to different concentrations of isomeric hydroperoxides stored at 25 C for 24 hr; before storage, the distribution of hydroperoxide was 78% 13-LOOH, *cis,trans;* 3% 13-LOOH, *trans,trans;* 18% 9-LOOH. At 15 mg/ml, a 1:1 ratio of positional isomers (13 and 9) existed; whereas, the predominant isomer at 50 mg/ml was the 9-LOOH. The 13-LOOH geometrical isomer approached a 1:1 ratio at 50 mg/ml. Rate of isomerization increased as total concentrations of hydroperoxides were increased during storage. Therefore, to minimize isomerization, hydroperoxides should be stored in as dilute solution as possible.

Of the conditions tested, oxygen tension and pH had the most pronounced effects on isomeric hydroperoxides. Limited oxygen and alkaline pH shifted the ratio to about 1:1 for positional isomers. Other factors such as temperatures of reaction and of extraction, and choice of extractant caused minor shifts in the ratios of isolated isomeric hydroperoxides. In order to determine the product specificity of lipoxygenase-linoleic acid reaction mixtures, the following conditions are recommended: (a) incubate below 25 C, (b) do not prolong reaction time, (c) extract with $CHCl_3$:CH_3OH (2:1) for thorough extraction, (d) analyze as soon as possible, and (e) store samples at -20 C in a dilute solution. Results of these studies indicate the importance and need for standardized conditions in the work-up, manipulation, and storage of isomeric hydroperoxides for determination of product specificity of lipoxygenase. Only under carefully controlled environmental conditions can isomeric specificity of lipoxygenase be determined and reproducible results obtained.

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