# Lipoxygenase-Oxidized Soap Stock as Source of Hydroxy Conjugated Octadecadienoic Acids[1]

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## ABSTRACT

A laboratory procedure was developed for the production of hydroxy conjugated octadecadienoic acids (HOCD). Lipoxygenase oxidation of distilled or crude soap stock produces hydroperoxy conjugated octadecadienoic acids, which can be reduced to HOCD. Crude aqueous soy flour extracts and defatted soy flour are convenient, effective and inexpensive lipoxygenase sources. This new procedure allows high substrate concentrations of 100 mg/ml to be used while achieving 75% to 90% oxidation of the available linoleic acid during a reaction time of 20 to 40 min. Aqueous ethanol and dimethyl sulfoxide (DMSO) solvent systems were compared. The DMSO system produced higher yields of hydroperoxy acids with low enzyme concentrations. Hydroperoxide yields are good when short reaction times, high pH levels and free radical scavengers are used.

## INTRODUCTION

Potential industrial chemical outlets for hydroxy conjugated dienes (HOCD) are hydraulic fluids, lubricants, emulsifiers, plasticizers and coatings. The availability of low-cost soap stocks prompted the investigation of lipoxygenase (E.C. 1.13.1.13) oxidation as the first step in determining whether soap stocks could be used as a source of HOCD.

Lipoxygenase oxidation of the linoleic acid present in saponified vegetable fats produces 9(*R*)-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid and 13(*R*)-hydroperoxy-*cis*-9,*trans*11-octadecadienoic acid in about a 3 to 7 ratio (1,2). These hydroperoxy conjugated octadecadienoic acids are easily reduced to the corresponding hydroxy acids by a variety of methods. Chemical reduction of the hydroperoxides with triphenylphosphine, sodium bisulfite, stannous chloride, potassium iodide, sodium borohydride or hydrazine is a convenient way to prepare hydroxides (3,4). Mild catalytic reduction with nickel will also yield hydroxy acids (3).

Lipoxygenase oxidation of linoleic acid has long been used as an analytical assay method. The normal procedure requires a dilute substrate solution containing 2 x $10^{-3}$ to 2 x $10^{-4}$ g (7 x $10^{-6}$ to 7 x $10^{-7}$ moles) of linoleic acid per milliliter and ca. 10 $\mu$g/ml of purified enzyme (5-7). If a preparative laboratory procedure or industrial reaction based on the analytical lipoxygenase assay method is to be developed, an increase in substrate concentration and the use of a low-cost lipoxygenase source would be desirable.

The laboratory procedure reported in this paper gives up to 90% oxidation of available linoleic acid in solutions containing 10% soap stock. Defatted soy flour and crude aqueous soy flour extracts were used as effective lipoxygenase sources.

## MATERIALS

### Lipoxygenase Sources

Purified lipoxygenase was purchased from Nutritional Biochemical Corp. (NBC) and had an activity of 14,000 units/mg.

Defatted soy flour was prepared in the laboratory by cracking 1.2 kg of 1968 certified Hawkeye soybeans (10-30 mesh). The cracked beans were extracted by mixing with 2 liters of hexane and allowing the mixture to stand for 1 hr. The hexane was then decanted, 1 liter of hexane added and the mixture allowed to stand for 72 hr. The hexane was then removed by filtration through a Buchner funnel and the meal dried under vacuum. The dried meal was ground to a flour in a hammer mill and extracted three times with 1.5 liter portions of hexane. The mixture was set aside for 4 hr after each addition of hexane and then filtered. After drying the residue under vacuum, approximately 1 kg of defatted soy flour was recovered and stored at 3 C until needed for use per se or in making the aqueous soy flour extract.

Crude aqueous soy flour extracts were freshly prepared when needed by extracting defatted soy flour with 0.05 M (pH 10) borate-KOH buffer. The general procedure involved stirring 4 g of defatted flour with 40 ml of buffer for 5 min and then centrifuging at 10,000 rpm for 2 min. About 25 ml of clear extract could be decanted from the sediment. This crude extract was further purified for use in the standard lipoxygenase assay method by adding 2.5 ml of a 64 mg/ml $CaCl_2$ solution, centrifuging at 10,000 rpm for 3 min and decanting the clear extract. The activity of the $CaCl_2$-purified soy flour extract was compared with NBC-purified lipoxygenase by using the standard lipoxygenase assay procedure (5,6). Comparison of crude aqueous soy flour extract and $CaCl_2$-purified soy flour extract demonstrated that they were almost identical in activity. The crude aqueous soy flour extract was normally used in the scaled-up reactions.

### Substrates

Safflower acids (SFO) containing 75% linoleic acid were prepared by saponifying safflower triglycerides and then distilling the free acids at reduced pressure. SFO soaps were prepared by neutralizing the free acids with 1 to 1.5 N sodium or ammonium hydroxide.

A sample of undistilled acidulated soybean (SBO) soap stock from Swift & Co. contained 88% free fatty acids of which 52% was linoleic acid. The sample also contained 12% unsaponified material which consisted of phospholipids, mono-, di- and triglycerides. The crude SBO soaps were prepared by neutralizing the acids with 1 to 1.5 N sodium, potassium or ammonium hydroxide.

### Miscellaneous

Antifoam "B" came from Dow Corning Corporation; $\alpha$-tocopherol, from Distillation Products Industries.

## METHODS

### Lipoxygenase-Oxidation Procedure

The general oxidation procedure was as follows: A weighed amount of the fatty acids (5 g) was dissolved in a measured amount (10 ml) of dimethyl sulfoxide (DMSO) or 95% ethanol. The fatty acids were then neutralized by using a 5% to 10% molar excess of 1 to 1.5 N ammonium, potassium or sodium hydroxide. The base was added slowly with vigorous stirring. As the solution became viscous

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during the addition of the base, ca. 5 ml of pH 10 buffer was added to thin the mixture. Next, a measured amount of 0.05 M borate, phosphate or ammoinium chloride-ammonium hydroxide buffer of the desired pH was added. The solution was stirred for 15 to 30 min under a stream of oxygen. Stirring was stopped and the lipoxygenase source added. The manometric system was checked for leaks, the magnetic stirrer started at 80% to 90% of full speed and the rate of oxygen uptake recorded. To achieve high yields of hydroperoxide, the oxygen must be thoroughly mixed with the reaction mixture. The amount of substrate, solvent, buffer and enzyme used was varied to determine their effect on the reaction.

The reaction was stopped by adding enough 95% ethanol to increase the reaction volume 20%. The hydroperoxides in the reaction mixture were reduced with an equal molar amount of $NaBH_4$. Addition of diethyl ether helps control foaming during the reduction with $NaBH_4$. The solution was stirred for 1.5 hr after addition of $NaBH_4$ and then filtered through Celite in those experiments where defatted soy flour had been used as the enzyme source. No filtration was necessary when aqueous soy flour extracts were used. The reduced solution was acidified to pH 3 with dilute HCl and extracted four times with diethyl ether. An initial emulsion was usually formed and was broken by adding diethyl ether, petroleum ether and additional ethanol. The ether extracts were washed until neutral and dried over sodium sulfate. The ether was evaporated under reduced pressure and the final traces of solvent were removed under a stream of nitrogen. Actual recoveries were usually 90% to 95% of the total soap stock added unless some of the hydroperoxides decomposed before $NaBH_4$ reduction.

In some experiments results were similar when chloroform or *n*-butyl alcohol was used to extract the oxidized fatty acids from the reaction mixture.

### Oxidation of Linoleic Acid

A sample of 2 g linoleic acid (Hormel) was mixed with 4 ml of DMSO and 15 ml of potassium borate buffer (pH 10). A total of 4 ml of 2 N potassium hydroxide was added and the mixture stirred until clear. The potassium linoleate solution was stirred under an atmosphere of $O_2$ for 10 min and then 15 ml of clear soy flour extract prepared from 2 g of defatted soy flour was added. The solution was vigorously stirred and $O_2$ uptake monitored. BHT (2 mg in 0.25 ml DMSO) was added after 4 min. The reaction was stopped after 15 min by adding 40 ml methanol and the hydroperoxides reduced by stirring with 150 mg of $NaBH_4$ for 15 min. The mixture was then acidified with dilute HCl, 40 ml of $H_2O$ added and extracted three times with 30 ml portions of 3:1 chloroform-methanol. The extract was dried over sodium sulfate and the solvent evaporated. A total of 2.1 g of material was recovered.

Gas liquid chromatographic (GLC) analysis of an esterified and silylated sample showed 55% methyl linoleate and 45% methyl hydroxy conjugated octadecedienoate.

### Manometric System

An automatic gas-pressure controller and volume recorder was used to measure and record the rate of oxygen uptake (8).

### UV Analysis

UV spectra of the isolated, reduced, reaction products in 95% ethanol were obtained with a Cary-14 recording spectrometer. The $\lambda_{max}$ for hydroxy *cis,trans*-conjugated octadecadienoic acids (HOCD) is 233 nm. The per cent HOCD in the samples was calculated by using 27,063 as the extinction coefficient for HOCD (9).

UV analysis of the products from reactions carried out in aqueous DMSO were of little value because traces of DMSO in the extracted sample strongly absorbed in the same region as did the hydroxy conjugated diene. UV analysis of the reaction products from crude SBO soaps also presented a problem. The crude SBO soaps contained a few per cent conjugated diene plus unknown impurities, which had high extinction coefficients in the 233 nm region. The absorptivity of the unoxidized crude SBO acids were subtracted from the absorptivity of the oxidation product, but this procedure is not very accurate since the workup and extraction of the oxidized SBO acids remove varying amounts of the impurities present in the unoxidized SBO acids. GLC analysis was used whenever UV analysis was unsatisfactory.

### GLC Analysis

An Aerograph model A-350 fitted with a glass injector port and thermoconductivity detector served for GLC analysis of the silylated reaction products (10). A 0.25 in. x 8 ft column packed with 10% EGSS-X on 100/120 mesh Gas-Chrom P was used for separating the silylated fatty acids. The GLC was operated under isothermal conditions at 165 C and a flow rate of 80 to 100 ml/min of helium.

The fatty acids were silylated with bis(trimethylsilyl) acetamide (BSA). The silylation procedure involved addition of 10 $\mu$l of dry pyridine to 10-12 $\mu$l of fatty acid in a 0.5 dram screw-capped vial followed by 50 $\mu$l of BSA. The vial was then capped and the solution warmed to 45-50 C for 20 min. The silylated oxidized SFO acids were completely separated, except for stearic and oleic acids, which were only partially separated and consequently integrated as one peak. The silylated oxidized SBO acids contained about 5% each of conjugated dienoic acids, linolenic acids and hydroxy-octadecatrienoic acids containing a conjugated dienol system. These silylated acids were only partially separated from HOCD.

Analysis of the silylated reaction products agreed to within ±0.5% of the GLC analysis obtained by methylating the reaction products with diazomethane.

## RESULTS

### Activity of Enzyme Preparations in Ethanol

Soybean flour is the richest source of lipoxygenase currently known (11). Three soybean lipoxygenases of different purities were investigated. The activities of NBC-purified lipoxygenase, aqueous soy flour extract and defatted soy flour were compared.

The NBC-purified lipoxygenase had an activity of 14,000 units/mg when used in the standard lipoxygenase assay procedure (5,6). One milliliter of $CaCl_2$-purified aqueous soy flour extract had an activity equal to 0.25 mg (3500 units) of purified lipoxygenase when analyzed by the standard lipoxygenase assay method. This value for the aqueous soy flour extract agrees with the activity calculated from published data (12). From these data, 1 g of defatted soy flour was calculated to contain the equivalent of 2.5 mg (35,000 units) of NBC-purified lipoxygenase. The substrate to enzyme (S/E) ratios are defined here as milligrams of substrate per 14,000 units of lipoxygenase (1 mg purified lipoxygenase was equivalent to 14,000 units).

When equivalent units of purified lipoxygenase and aqueous soy flour extract were used in reactions having substrate concentrations of 4.0 mg and 100 mg/ml, rather than the 0.1 to 0.2 mg/ml used in the lipoxygenase assay method, the aqueous soy flour extract and the defatted soy flour had a much higher activity than the purified lipoxygenase. Figure 1 shows the oxidation of distilled SFO soap stock in 10% ethanol and demonstrates the relative activity of the three lipoxygenase sources. In Figure 1 the soap stock concentration was 4 mg/ml and an S/E ratio of 80 was selected. Under these conditions, the aqueous soy flour extract activity was extremely high. In other experiments

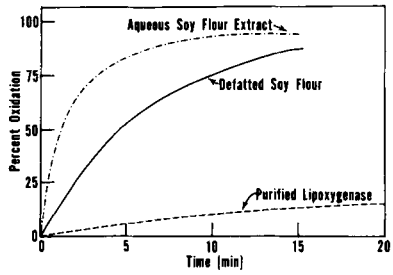


FIG. 1. Activity of various lipoxygenase sources in 10% ethanol. Substrate, Safflower (SFO) soaps; substrate concentration, 4 mg/ml; substrate to enzyme (S/E) ratio, 80.

where the substrate concentration and the S/E ratio were increased, the activity of aqueous soy flour extract was similar to that of defatted soy flour.

The difference in activity between the aqueous soy flour extract and the purified lipoxygenase was the most striking. Purified lipoxygenase was relatively inactive and easily inhibited when S/E ratios of 80 or higher were used. In experiments where S/E ratios were 10 to 20, the purified lipoxygenase appeared to be much more active and gave reaction rates similar to those obtained with aqueous soy flour extracts.

Figure 2 shows the oxidation rate of SFO soaps in 10% ethanol at various substrate concentrations with defatted soy flour as the enzyme source. The fatty acid concentrations were varied from 4 to 100 mg/ml and the S/E ratio was held constant at 200. Under these conditions, the per cent oxidation and the reaction rates decreased as the substrate concentration was increased.

Higher than 10% ethanol concentrations rapidly denatured the enzyme and caused low reaction rates. Less than 5% ethanol concentration gave poor substrate solubilities and low reaction rates at high SFO soap concentrations.

## Activities of Enzyme Preparations in DMSO

The oxidation of SFO soaps in 20% DMSO by various enzyme preparations is plotted in Figure 3. In these reactions, the substrate concentration was 100 mg/ml and the S/E ratio 500.

As in the ethanol reactions (Fig. 1), the defatted soy flour and the aqueous soy flour extracts were much more active than the purified lipoxygenase. Defatted soy flour appears to be somewhat more active than the aqueous soy flour extract when high substrate concentrations are used, but the difference may be due to a slightly higher enzyme concentration rather than to greater lipoxygenase activity.

The soy flour and aqueous soy flour extract oxidations in DMSO at high substrate concentration routinely produced 80% to 90% oxidation, whereas the purified

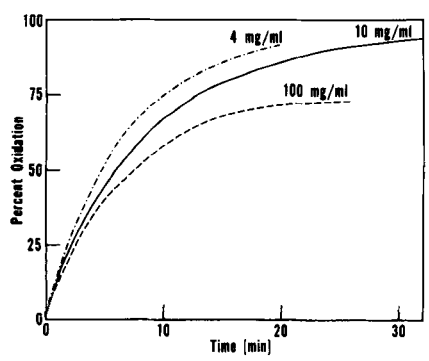


FIG. 2. Lipoxygenase activity of defatted soy flour as function of SFO soap concentration in 10% ethanol. S/E ratio, 200.

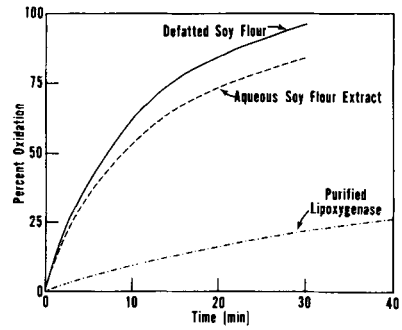


FIG. 3. Activity of various lipoxygenase sources in 20% dimethyl sulfoxide (DMSO). Substrate, SFO soaps; substrate concentration, 100 mg/ml; S/E ratio, 500.

lipoxygenase oxidations were not reproducible and stopped at 30% oxidation or less. Oxidation rates in 10% DMSO were only slightly less than those involving 20% DMSO. However, 30% DMSO caused rapid denaturing of the enzyme and consequently gave lower reaction rates and less total oxidation.

Oxidation rates of SFO soaps in 10% ethanol and 20% DMSO with defatted soy flour are compared in Figure 4. In this comparison, soap stock concentrations were 100 mg/ml and S/E ratios 1000. Surprisingly, the reaction containing DMSO outperformed the reaction containing ethanol by about 30% and clearly demonstrated the advantage of using DMSO in reactions involving high soap stock concentrations and small amounts of enzyme. Other polar aprotic solvents, such as tetrahydrofuran, pyridine and acetone, were tried in place of DMSO, but these solvents severely inhibited lipoxygenase oxidation.

The effect of varying the S/E ratio on the oxidation rate of SFO soaps in 20% DMSO is seen in Figure 5. Close to the maximum reaction velocity was obtained when 2 g of soy flour was used with 5 g of SFO soaps (S/E ratio = 1000). When S/E ratios were more than 1000, the rate of oxygen uptake was proportional to the enzyme concentration. The maximum reaction velocity is usually reached by increasing the substrate concentration until the substrate saturates the active sites on the enzyme. However, experiments with an S/E ratio of 667 and 1000 indicate that the substrate concentration is already a limiting factor. Apparently increasing the substrate concentration to an S/E ratio of more than 1000 inhibits and denatures the enzyme. This inhibition is probably due to impurities or to fatty acids other than linoleic acid since more enzyme (Fig. 6) was needed in the SBO soap oxidations than in the SFO soap oxidations to achieve the same degree of reaction.

The reaction rate also influenced the extent of oxidation. Unless a reaction rate close to the maximum was selected, the total yield of hydroperoxy conjugated dienoic acid was decreased. Although these results are not fully understood, they may be related to the rate at which the lipoxygenase is being denatured or the manner in which it is inhibited. Not enough work has been completed on this aspect to sustain an explanation. The stirring rate was varied to determine if oxygen was the limiting factor, but the reaction rate did not vary when various methods and rates of stirring were used, provided they were vigorous enough to produce a foamy solution equal to at least about twice the initial volume of the reaction mixture.

## Product Composition From SFO Soap Oxidations

Conjugated-hydroperoxy acids formed during oxidations of linoleic acid in SFO were first reduced to the HOCD by $NaBH_4$. The isolated reaction product was analyzed for hydroxy *cis,trans*-conjugated diene by UV spectroscopy. A total analysis of the silylated acids was determined by gas

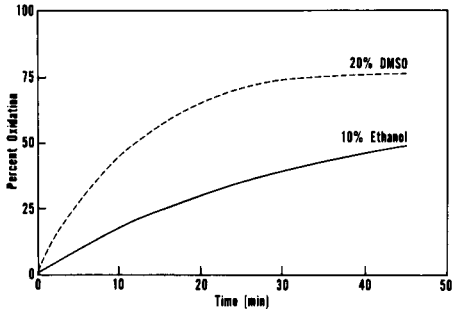


FIG. 4. Comparison of lipoxygenase oxidation rates in 10% ethanol and 20% DMSO. Enzyme source, defatted soy flour; substrate, SFO soaps, Substrate concentration, 100 mg/ml; S/E ratio, 1000.

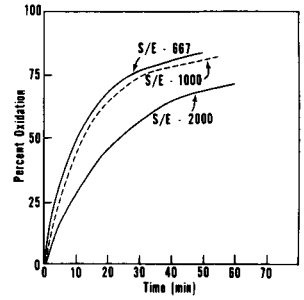


FIG. 5. Effect of varying substrate to lipoxygenase (S/E) ratio in 20% DMSO. Enzyme source, defatted soy flour; substrate, SFO soaps; substrate concentration, 100 mg/ml.

chromatography.

Reportedly, oxooctadecadienoic acid can also be produced by lipoxygenase action (13) but since $NaBH_4$ was used as the reducing agent here, any oxooctadecadienoic acid present could be reduced to HOCD and would be included in the GLC analysis as HOCD. Random checks on the oxidation products by UV analysis before $NaBH_4$ reduction did not, however, indicate the presence of oxooctadecadienoic acid ($\gamma_{max}$ = 278 nm) although concentrations of ca. 1% may have escaped detection.

Oxidations carried out in 20% DMSO using various enzyme preparations are reported in Table I. Total recoveries were usually 85% to 95%. In some experiments, the combined percentages of linoleic acid plus HOCD from Table I do not equal the 75% linoleic acid present in SFO acids. This discrepancy is due to two problems which resulted in low values for the per cent HOCD. The first problem arises because HOCD is more soluble in the aqueous layer than the other fatty acids, a condition which makes complete extraction of the HOCD difficult. The loss of HOCD by polymerization and decomposition of the hydroperoxy conjugated diene to aldehyde and aldehyde-acid fission products, the second problem (4,14,15), probably accounts for most of the differences noted in Table I. Evidence for hydroperoxide decomposition was indicated by increased percentages of palmitic, stearic and oleic acids, as well as by detection of azelaic aldehyde which would result from cleavage of the carbon-carbon bond at the 9 position.

Antioxidants are thought to control both carbon-carbon fission of the hydroperoxide and free radical initiated polymerization (4,14,16). Although some antioxidants inhibit lipoxygenase activity, others appear to have little or no effect (1,11). The data in Table II demonstrate that incorporating antioxidants in lipoxygenase oxidations containing high substrate concentrations of SFO soaps can improve the yield of hydroperoxides, probably by reducing fission of the carbon-carbon chain in polymerization. Antioxidants other than butylated hydroxy toluene (BHT) and α-tocopherol could undoubtedly be used if they do not severely inhibit lipoxygenase activity.

Based on product composition, the percent oxidation was higher in the experiments containing antioxidants, but the total $O_2$ uptake was less than in control experiments containing no antioxidants. The reduction in total $O_2$ uptake may be due to prevention of autoxidation and hydroperoxide decomposition to aldehydes and aldehyde acids by free-radical initiated reactions and secondary oxidation of the aldehydes (4,16). An alternate explanation for the increased yield of HOCD in Tables II and III is that antioxidants may protect lipoxygenase by destroying lipid free-radical fragments before they react with lipoxygenase. This explanation may be the most plausible since antioxidants reportedly produce hydroperoxide decomposition (17,18) which would actually reduce the HOCD yield. The observed increase in HOCD yields must then be due to prolonged enzyme activity, which results in a higher percent oxidation. The optimum antioxidant concentration necessary to suppress fragmentation and loss of the hydroperoxide was not determined.

Solutions had to have a pH of 9 to 10 before good yields of hydroperoxide were obtained. The high pH presumably reduces the activity of a reported lipohydroperoxidase, which catalyzes hydroperoxide decomposition (15). The yield of HOCD from reactions in solutions having a pH of 7 to 8 reached a maximum of 70-80% and then decreased rapidly due to loss of hydroperoxide.

Chemical reduction of the hydroperoxides in situ during the oxidation reaction with triphenylphosphine or hydrazine proved an impractical method of preventing hydroperoxide decomposition because these reducing agents rapidly denatured the enzyme.

### Oxidation of Crude SBO Soap Stock

Crude SBO soap stock in 20% aqueous DMSO was oxidized with defatted soy flour or aqueous soy flour extracts as the lipoxygenase source. Oxidation of soybean soaps was explored since the large supply and ready availability of these soaps make them attractive for an industrial scale operation. The enzymatic oxidation of crude SBO soaps followed the same general reaction patterns that were established with distilled SFO soaps. The main difference was that the SBO oxidations were completed in a shorter reaction time since SBO soaps contain less linoleic acid than SFO soaps.

Some typical oxidations of crude SBO soaps with defatted soy flour and soy flour extracts as the enzyme source are plotted in Figure 6. As was observed with SFO soaps, the oxidation rates are about the same when either soy flour or soy flour extracts are the lipoxygenase source. Addition of 0.75% BHT slightly reduces the total amount

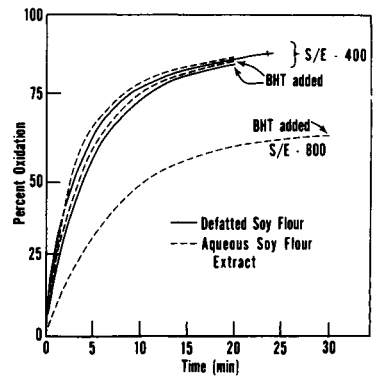


FIG. 6. Effect of butylated hydroxy toluene (BHT) and S/E ratios on lipoxygenase oxidation of crude soybean oil soap stock in 20% DMSO. Enzyme sources, defatted soy flour and aqueous soy flour extract; substrate concentration, 100 mg/ml.

TABLE I

Analysis of Oxidized SFO[a] in 20% DMSO[b]

| Enzyme preparation | S/E | HOCD, % | Lo, % | Oxidation,[c] % | Total recovery, % |
|---|---|---|---|---|---|
| Purified lipoxygenase | 250 | 12.5 | 63 | 15 | 93 |
| Soy flour extract | 500 | 48 | 18 | 76 | 90 |
| Defatted soy flour | 500 | 59 | 7.3 | 90 | 90 |

[a]SFO, safflower oil; DMSO, dimethyl sulfoxide; S/E, substrate to enzyme ratio; HOCD, hydroxy conjugated octadecadienoic acids; Lo, linoleic acid.
[b]Concentration of soap stock, 100 mg/ml of 20% DMSO. Reaction time, 45 min.
[c]Per cent oxidation is based on the per cent linoleic acid remaining in the sample.

of oxygen uptake as had been observed with SFO soaps. Again, product analysis indicated that about the same percentage of linoleic acid was oxidized regardless of whether BHT was added. One half as much enzyme was used in the experiment employing an S/E ratio of 800 (Fig. 6) as was used in the other experiments and shows the effect of enzyme concentration on the reaction rate. The oxidations with crude soy flour extract had slightly higher initial reaction rates, but these reactions were more easily inhibited or denatured than the oxidations using defatted soy flour.

In general, it was necessary to use more enzyme with the crude SBO soaps than with the SFO soaps to get 80% to 90% oxidation of the available linoleic acid. More enzyme was probably required because the 7.6% isolated *trans* and 4.3% conjugated diene fatty acids present in the crude SBO soaps inhibited some of the enzyme. Conjugated 10,12-octadecadienoic acid is known to be a particularly effective competitive inhibitor for lipoxygenase (6).

### Product Composition From SBO Soap Oxdidations

Analysis of the reaction mixture from oxidation of SBO soaps in 20% DMSO are given in Table III. The reaction products were treated in the same manner as described for the SFO oxidation products and then analyzed by UV and GLC.

In general, actual recoveries were good and 70% to 90% oxidation was normal. Shorter reaction times for the oxidation of SBO soaps resulted in less hydroperoxide decomposition than in the SFO soap oxidations. However BHT was still necessary to obtain maximum HOCD yields. About 50% of the linolenate present in SBO soaps was removed during oxidation. Quantitative GLC analysis of the remaining linolenic acid and its oxidation products was difficult because the small peaks for these acids were poorly separated from the large HOCD peak.

### Other Reaction Parameters

Reaction parameters such as temperature, stirring rate, stirring efficiency, buffer pH, counter ions used in soap formation, micelle size and surface tension are all factors to be considered in obtaining an optimum reaction rate. These parameters were given only a cursory study to determine if they could cause any major changes in reaction rates. Most of them have been adequately studied in lipoxygenase oxidations where purified lipoxygenase and low substrate concentrations were used. Thus it was only necessary to confirm that at high substrate concentration, lipoxygenase oxidations follow the same patterns reported for oxidations at low substrate concentrations (1,7,11,12,19,20).

The optimum reaction temperature was 25-30 C. Reactions run at 0 and 15 ± 2 C were sluggish while reaction temperatures of about 40 C gave low yields of HOCD because of rapid enzyme inhibition or denaturation. Stirring needed to be vigorous enough to produce a foamy solution. In reactions where antifoam "B" was added to prevent foaming, the reactions were sluggish and quickly became inhibited. This observation is in direct contrast with published data on dilute substrate solutions, which indicate a foamy solution should be avoided since foaming rapidly denatures lipoxygenase (19).

Phosphate, borate and ammonium hydroxide-ammonium chloride buffer systems were used. The different counter ions had no observable effect on the reaction rates except that reaction mixtures having a buffered pH of 9 to 10 gave the best yields of hydroxy conjugated dienes. The rate of oxygen optake did not depend on the pH of the reaction mixture, but when pH levels were low, extensive decomposition of the hydroperoxy conjugated dienes occurred. Also high pH levels ensure the neutralization of all the fatty acids and help to improve the dispersion of the soaps. The optimum pH level is in agreement with the results generally reported (1). As mentioned, high pH"s increase yields because high pH values are believed to inhibit a lipohydroperoxide breakdown factor present in soybeans (15).

Potassium, sodium and ammonium hydroxide solutions were used to neutralize the fatty acids in preparing the soap stocks. The different bases do affect the solubility of the

TABLE II

Effect of Antioxidants on Oxidized SFO Soap Composition and Yields[a]

| Antioxidant | Enzyme source | Lo, % | HOCD, % | Oxidation,[b] % |
|---|---|---|---|---|
| None | Soy flour | 22 | 48 | 71 |
| 0.3% α-Tocopherol | Soy flour | 20 | 52 | 74 |
| 0.75% BHT[c] | Soy flour | 12 | 64 | 84 |
| None | Soy flour extract | 19 | 52 | 75 |
| 0.75% BHT | Soy flour extract | 13 | 63 | 83 |

[a]Data from oxidation of SFO soap in 20% DMSO for 45 min at a concentration of 100 mg/ml and pH levels of about 9.
[b]Per cent oxidation based on per cent linoleic acid left in sample.
[c]BHT, butylated hydroxy toluene.

TABLE III

Analysis of Oxidized SBO[a] Soaps

| Antioxidant added | Enzyme source | Reaction time, min | Lo, % | HOCD, % | Actual recovery, % | Oxidation,[b] % |
|---|---|---|---|---|---|---|
| 0.0% (Control) | Soy flour | 25 | 6.0 | 36.0 | 88.5 | 88 |
| 0.75% BHT | Soy flour | 18 | 5.9 | 43.8 | 96.0 | 88 |
| 0.0% (Control) | Soy flour extract | 20 | 5.9 | 44.6 | 96.0 | 88 |
| 0.75% BHT | Soy flour extract | 18 | 4.3 | 47.2 | 93.0 | 91 |

[a]SBO, soybean soap concentration of 100 mg/ml of 20% DMSO.
[b]Per cent oxidation based on per cent linoleic acid left in sample.

soaps, but the oxidation rates were not noticeably influenced. Calcium chloride reportedly enhances the oxidation rate when purified lipoxygenase is used (21). Addition of various amounts of calcium chloride to crude aqueous soy flour extracts had no effect on oxidation rate.

The proper preparation of the substrate solution was important. For 80-90% oxidations, the soap stock emulsions had to be well dispersed before the enzyme was added to the solution. If the substrate solutions were properly prepared, reactions with aqueous soy flour extracts or soy flour were reproducible from day to day. When antioxidants were used, the per cent oxidation calculated from total oxygen uptake agreed to within ±3% of the per cent oxidation calculated from UV and GLC analysis.

## DISCUSSION

One purpose of this paper is to show that lipoxygenase oxidation of linoleic acid in soap stock can be scaled up to produce reasonably complete oxidation in a short time. We demonstrated that oxidation was effective on crude SBO soap stocks using crude aqueous enzyme extracts isolated from defatted soy flour. The problem of hydroperoxide decomposition during the reaction was controlled by using pH 10 buffers and minimum reaction times. The addition of an antioxidant also increased the yield of hydroperoxide. The reason a foamy solution was necessary is probably to provide a large surface area so that oxygen can be efficiently transported to the enzyme surface.

The large difference between the activity of purified and crude lipoxygenase when used in reactions containing high substrate concentration was unexpected. Lipid hydroperoxides and hydroperoxide radicals are known to react with the thio groups present in protein (22), which may explain why purified lipoxygenase is denatured more rapidly than crude lipoxygenase preparations. The cruce lipoxygenase sources contain a large amount of inactive protein that could also react with the lipid hydroperoxide radicals and consequently protect the lipoxygenase from being denatured.

An alternate explanation may be that lipoxygenase requires a coenzyme or a protein subunit for prolonged action. This protein unit could be lost during purification and may account for the differences in the activities of the lipoxygenase preparations.

Our procedure represents a laboratory method for preparing hydroxy conjugated octadecadienoic acid concentrates suitable for small scale investigations. This method for the preparation of hydroxy conjugated acids should be of synthetic value since a wide variety of reactions are possible starting with the hydroxy conjugated diene functionality.

A second purpose of this paper is to call attention to the economics of lipoxygenase oxidation of soap stock. Crude soybean soap stock ($0.06/lb) is an economical source of sodium linoleate and the aqueous soy flour extract is not an expensive lipoxygenase source. DMSO is a relatively expensive solvent for industrial use, but it is not consumed in the reaction. Consequently, high recoveries (98%) of the DMSO from the reaction mixture have been achieved. These factors suggest that the cost of commercially produced HOCD may be low enough to compete with similar organic intermediates. The possibility of scaling up this reaction for potential industrial use is being studied.

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