Formation of Conjugated Diene and Triene Products in Lipoxygenase Oxidation of C18, C20, C22 PUFAs

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The determination of conjugated diene formation revealed that the mol % conversions of all *cis*-6,9,12-octadecatrienoic acid [γ-linolenic, 18:3(n-6)], all *cis*-5,8,11,14-eicosatetraenoic acid [arachidonic, 20:4(n-6)], all *cis*-5,8,11,14,17-eicosapentaenoic acid [20:5(n-3)], and all *cis*-4,7,10,13,16,19-docosahexaenoic acid [22:6(n-3)] into conjugated diene products by soybean lipoxygenase-1 at pH 9.0 were 84, 86, 60 and 40% of that of all *cis*-9,12-octadecadienoic acid [linoleic, 18:2(n-6)], respectively. On the other hand, the conversions of all *cis*-9,12,15-octadecatrienoic acid [α-linolenic, 18:3(n-3)], all *cis*-5,9,12-octadecatrienoic acid (c5,c9,c12-18:3), and *trans*-5,*cis*-9,*cis*-12-octadecatrienoic acid (t5,c9,c12-18:3) were equal to that of 18:2(n-6). The lowering of the conjugated diene formation in the oxidation of 18:3(n-6), 20:4(n-6), 20:5(n-3), and 22:6(n-3) by the lipoxygenase was thought to be caused by the further oxidation of conjugated diene monohydroperoxides to yield conjugated triene products. For this reason, the conventional lipoxygenase method gave erroneous values for *cis,cis*-methylene interrupted polyunsaturated fatty acids (PUFA) in oils containing a large amount of 20:5(n-3) and 22:6(n-3) such as fish oils. However, by changing the pH of reaction mixtures from 9.0 to 11.0, the secondary oxidation of conjugated diene monohydroperoxides was completely inhibited, and the PUFA values in fish oils obtained by this improved method were in good agreement with those obtained by a GLC method.

Soybean lipoxygenase catalyzes the oxidation of PUFA containing a *cis,cis*-1,4-pentadiene group to generate *cis,trans*-conjugated diene hydroperoxides (1-3). Holman et al. tested the substrate specificity of a number of PUFA for soybean lipoxygenase oxidation on the basis of the determination of conjugated diene formation (2). They showed that 18:2(n-6) was the best substrate, and that other fatty acids having the *cis,cis*-1,4-pentadiene group were less reactive.

Bild et al. found that 20:4(n-6) can be oxidized by soybean lipoxygenase-1 to produce 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic acid via a conjugated diene monohydroperoxide (4). They have also predicted that all substrates which contain the n-6,9,12 system of unsaturation in the *cis* configuration should be susceptible to double dioxygenation by lipoxygenase-1 (5). These results suggested that differences in the mol % conversion of PUFA into conjugated diene hydroperoxides may be due mainly to the differences in the secondary oxidative rates of conjugated diene products.

On the other hand, the lipoxygenase oxidation has been applied to the determination of *cis,cis*-methylene interrupted PUFA content of oil and fat products (6-9). PUFA content can be calculated from the UV absorption at 234 nm corresponding to conjugated diene. However, it is presumed that this method gives erroneous values for PUFA content of oils which contain a considerable amount of PUFA having the n-6,9,12 system of unsaturation because further oxidation of the conjugated diene products will occur.

In the present study, we investigated the substrate specificity for the formation of conjugated diene and triene products in the oxidation of a series of PUFA by the soybean lipoxygenase. In addition, an attempt is made to extend the enzymatic method for measuring *cis,cis*-methylene interrupted PUFA to a variety of oil and fat products.

MATERIALS AND METHODS

Preparation of pure fatty acids. Raw materials were safflower oil for 18:2(n-6), linseed oil for 18:3(n-3), sardine oil for 20:5(n-3) and, for 22:6(n-3), ether extracts from *Pinus koraiensis* seeds for c5,c9,c12-18:3 (10), and *Aquilegia akitensis* seeds for t5,c9,c12-18:3 (11), respectively. The mixed methyl esters obtained from each oil by transesterification with sodium methoxide were separated by silver nitrate-silicic acid column chromatography (12). Each purified methyl ester was saponified and then the unsaponifiable matter, such as tocopherols, was removed. After acidifying the solution, the free fatty acid fraction was extracted with ether. Preparations of 18:3(n-6) and 20:4(n-6) from their methyl esters were carried out by the method described above. Methyl esters of 18:3(n-6) and 20:4(n-6) were provided by Idemitsu Sekiyu Co., Ltd., and Lion Co., Ltd., both of Tokyo, Japan, respectively. The recovered fatty acid was refined by silicic acid column chromatography just before use. Each refined fatty acid gave only a single spot on TLC, and its methyl ester showed a purity of over 99% in GLC.

Purification of vegetable and fish oils. Olive, soybean and linseed oils were purchased from Nakarai Chem. Co.,

TABLE 1

Analysis of Oxidized Products by Determination of UV Absorption of Reaction Mixtures Containing Soybean Lipoxygenase and 3.3 × 10^{-6} mol of Fatty Acids at pH 9.0

Substrate	Mol % conversion of substrates into conjugated diene products[a]	Absorbance at 268 nm
18:2(n-6)	100	0.003[b]
c5,c9,c12-18:3	100	0.003[b]
t5,c9,c12-18:3	98	0.004[b]
18:3(n-3)	96	0.009[b]
18:3(n-6)	84	0.070
20:4(n-6)	86	0.068
20:5(n-3)	60	0.167
22:6(n-3)	40	0.273

[a]The mol % conversion represented as the relative percentage to 18:2(n-6) on the basis of conjugated diene formation per mol of substrate.
[b]No peak at 268 nm was detected.

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Kyoto, Japan. Safflower oil was obtained by hexane extraction from safflower seeds. Sardine, pollak-liver and cuttlefish-liver oils were obtained from industrial plants. These oils were refined by silicic acid column chromatography.

Oxidation procedure. Soybean lipoxygenase oxidations of substrates were carried out under the conditions of the method described by Madison and Hughes (9). This is a modification of the AOAC official method for the determination of PUFA.

For measuring *cis,cis*-methylene interrupted PUFA in vegetable and fish oils, the above method and an improved method were used. The experimental procedures of the improved method were the same except that the pH of the solution was adjusted to 11.0 instead of 9.0 (9) in the improved method.

Lipoxygenase solution. Soybean lipoxygenase (Sigma Type I, largely lipoxygenase-1, activity 126500 units/mg) was obtained from Sigma Chemical Co., St. Louis, Missouri.

An active lipoxygenase solution was prepared by dissolving 50 mg lipoxygenase in 500 ml of ice-cold 0.2 M borate buffer (pH 9.0 or 11.0). An inactive solution was obtained by holding the enzyme solution in boiling water for 30 min.

Substrate solutions. The fatty acid (3.3×10^{-4} mol) was dissolved with a small amount of 95% ethanol and made up to 10 ml with distilled water. The entire solution was transferred into a 100-ml volumetric flask containing 20 ml 1.0 M borate buffer (pH 9.0) and diluted to the mark of the flask with water.

Vegetable or fish oil (100 mg) was saponified overnight in a 20-ml reaction vessel with 5 ml alcoholic KOH (1.0 M). Saponification was carried out under an atmosphere of nitrogen in the dark. After saponification, 5 ml 1.0 M HCl was added. If the solution was not clear, 1.0 M KOH was added drop by drop until the solution became clear. The entire sample was transferred with hot distilled water into a 100-ml volumetric flask containing 20 ml 1.0 M borate buffer (pH 9.0 or 11.0).

Incubation. One ml substrate solution was pippetted into each of two 100-ml volumetric flasks. Nine ml inactivated enzyme solution was added to one flask, and nine ml activated enzyme solution was added to the other. An additional 10 ml distilled water was placed in both flasks, and they were placed in a 15 C constant temperature water bath for 30 min. Reaction mixtures were diluted to the mark with distilled water, and the ultraviolet (UV) spectra were determined with a Hitachi 124 spectrophotometer (Hitachi Seisakusho Co., Tokyo, Japan). The spectrophotometer was set to read zero absorbance with the blank solution (the solution containing the inactivated enzyme).

PUFA contents of vegetable and fish oils. Calculation of the *cis,cis*-methylene interrupted PUFA content of the oils was carried out on the basis of a standard curve, which was prepared using 18:2(n-6) (purity: above 99%).

Open tubular gas chromatographic analysis of fatty acid composition. Oils refined by silicic acid column chromatography were directly converted to methyl esters with 0.5 M sodium methoxide-methanol reagent. These esters were purified by TLC on a 0.5-mm layer of Silicagel G (Merck, Darmstadt, West Germany) by development with hexane/ether (8:2, v/v).

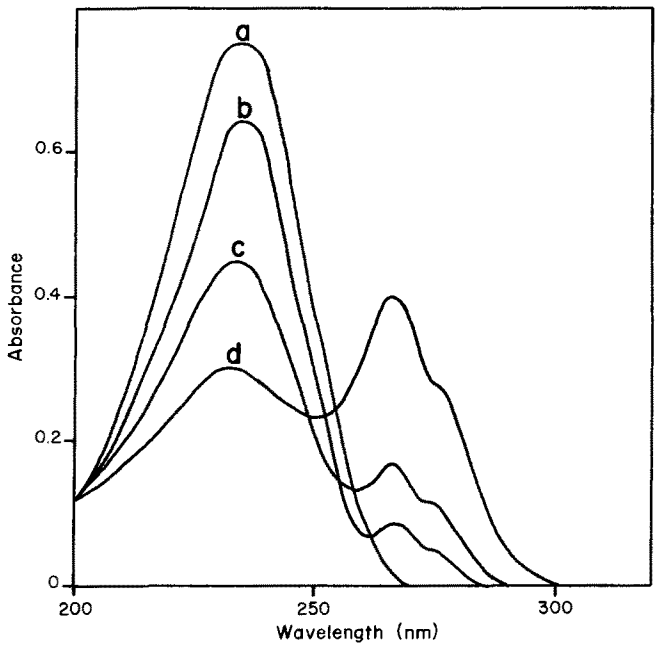

FIG. 1. UV spectra of reaction mixtures containing the lipoxygenase and fatty acids. a, 18:2(n-6), c5,c9,c12-18:3, t5,c9,c12-18:3, and 18:3(n-3); b, 18:3(n-6) and 20:4(n-6); c, 20:5(n-3), and d, 22:6(n-3).

Open-tubular GLC of the methyl esters was carried out with Shimadzu GC-6A and GC-7A instruments (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with dual FID detectors using wall-coated glass columns coated with SP-2300 or SP-2340 (Supelco Inc., Bellefonte, Pennsylvania) (50 m × 0.3 mm i.d.). Flow rate of the hydrogen carrier gas was 0.5 ml/min; the column temperature was 170 C, and the detector and injector temperatures were 230 C.

RESULTS AND DISCUSSION

Table 1 shows the mol % conversion of substrate fatty acids into conjugated diene products by the soybean lipoxygenase. The conversions are represented as the percentage relative to 18:2(n-6) on the basis of conjugated diene formation/mol of substrate. The isomers of 18:2(n-6) having a nonmethylene-interrupted olefinic bond (c5,c9,c12- and t5,c9,c12-18:3) showed mol % conversion approximately equal to that of 18:2(n-6). Therefore, the isolated *cis* and *trans*-5-olefinic bonds were presumed to have no influence on the lipoxygenase oxidation. The 18:3(n-3) also showed the same level of conversion into conjugated diene products by lipoxygenase as that of 18:2(n-6). However, the percentages of conversion of other PUFA were lower than that of 18:2(n-6).

The UV absorption spectra of certain reaction mixtures are illustrated in Figure 1. A maximum peak at 238 nm was observed in all substrates. This peak was considered to be due to *cis,trans*-conjugated diene monohydroperoxides. However, in the oxidations of 18:3(n-6), 20:4(n-6), 20:5(n-3) and 22:6(n-3) by the lipoxygenase, two or three other peaks corresponding to conjugated triene were observed in the range from 260 to 280 nm.

Bild et al. showed that soybean lipoxygenase-1 acted upon 20:4(n-6) to generate dihydroperoxy conjugated triene products (4,5). They also found the appearance of

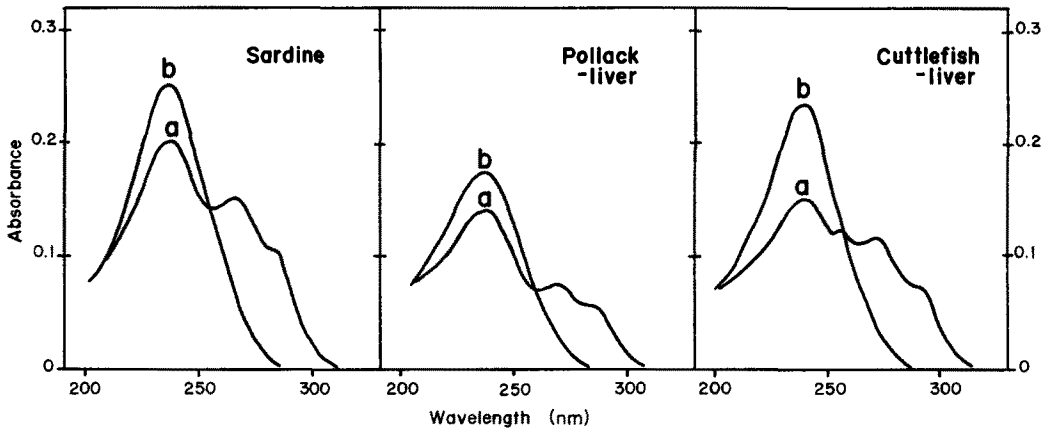

FIG. 2. UV spectra of reaction mixtures containing the lipoxygenase and saponified fish oils. Reactions were run at pH 9 (a) and 11 (b).

UV absorption corresponding to conjugated triene during the lipoxygenase-1 reaction of 18:3(n-6) and all *cis*-9,12,15-eicosatrienoic acid; therefore, they demonstrated that all substrates which contain the n-6,9,12 system of unsaturation in the *cis* configuration should be susceptible to dihydroperoxidation by the lipoxygenase-1. Soybean lipoxygenase (Sigma Type I, largely lipoxygenase-1) used in this study is considered to show only the specificity of lipoxygenase-1, because the lipoxygenase-1 catalyzes the oxidation of fatty acid substrates, but the lipoxygenase-2 exhibits little activity at pH 9.0 (13). Therefore, the lower amounts of conjugated diene formed in the oxidation of 18:3(n-6), 20:4(n-6), 20:5(n-3) and 22:6(n-3) can be attributed to the further oxidation of conjugated diene monohydroperoxides to yield conjugated triene products.

This assumption was confirmed by the results given in Table 1, which shows the absorbance at 268 nm of reaction mixtures containing lipoxygenase and fatty acids. The absorption at 268 nm corresponds to the conjugated triene. The highest absorbance value was observed in the oxidation of 22:6(n-3), followed by 20:5(n-3), 18:3(n-6), and 20:4(n-6). This order was the reverse of the mol % conversion of fatty acids into conjugated diene products. Since the monohydroperoxides formed in the oxidation of 18:3(n-6), 20:4(n-6), 20:5(n-3) and 22:6(n-3) by the lipoxygenase-1 have a doubly allylic methylene group on n-11 adjacent to a conjugated diene system (1-3), the conjugated triene products could be formed by the abstraction of hydrogen from this methylene group and oxidation at n-13. On the other hand, the absence of conjugated triene products in the lipoxygenase oxidation products from 18:2(n-6), c5,c9, c12-18:3, t5,c9,c12-18:3, and 18:3(n-3) is explained by the fact that there are no remaining doubly allylic methylene groups in the monohydroperoxide molecules formed in the oxidation of these fatty acids by the lipoxygenase-1.

Bild et al. (5) found that the pH optimum for the secondary oxidation of the conjugated diene hydroperoxide was around 7.5 and that lipoxygenase-1 exhibited very little activity on this reaction above pH 10.0. On the other hand, the pH optimum for the formation of conjugated diene hydroperoxide from PUFA by the lipoxygenase-1 reached a maximum at around 9.0 and persisted to pH 10.5 (5). After these findings were confirmed by the lipoxygenase oxidation of 22:6(n-3), 20:5(n-3) and 18:2(n-6) at pH 11.0, in which mol % conversion of 22:6(n-3) and 20:5(n-3) into conjugated diene products were 96 and 97% of that of 18:2(n-6) respectively, fish oils were oxidized. Figure 2 shows the UV absorption spectrum of the reaction mixtures of fish oils with the lipoxygenase at pH 11.0. Only a peak at 238 nm corresponding to conjugated diene appeared in all three cases. These spectra data indicated that the secondary oxidation of hydroperoxides by the lipoxygenase was inhibited by the changing pH of reaction mixture from 9.0 to 11.0. This change made it possible to apply the enzymatic method for measuring *cis,cis*-methylene interrupted PUFA to fish oils.

TABLE 2

***cis,cis*-Methylene Interrupted PUFA Content (wt % of Triglycerides) of Vegetable and Fish Oils; Comparison of Assay Methods**

Sample	Madisons' method[a] (a modification of AOAC method)	Proposed method[a]	GLC method
Vegetable oils			
Olive	5.7 ± 0.4	5.7 ± 0.1	5.7
Soybean	56.9 ± 0.1	57.1 ± 0.5	57.5
Safflower	77.5 ± 0.6	77.5 ± 0.7	77.2
Linseed	71.5 ± 0.6	71.6 ± 0.7	71.7
Fish oils			
Sardine	28.8 ± 0.6	35.3 ± 1.2	36.8
Pollack liver	18.7 ± 0.7	22.9 ± 0.4	23.1
Cuttlefish liver	19.9 ± 1.0	32.6 ± 0.5	33.0

[a]Average of triplicate analysis. Results expressed in *cis,cis*-methylene interrupted PUFA contents ± standard deviation.

Table 2 shows the comparison of assay methods for the determination of *cis,cis*-methylene interrupted PUFA in vegetable and fish oils. The values for *cis,cis*-methylene interrupted PUFA in vegetable oils found by the three different methods are in good agreement with each other, and these PUFA values in fish oils were also in fairly good agreement with the GLC method and our proposed method. However, agreement was poor between values for these PUFA in fish oils obtained by the GLC analysis and by Madison and Hughes' method (9).

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