Substrate Specificity of Soybean Lipoxidase

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SUMMARY

Purified soybean lipoxidase was used to test the substrate specificity of all cis,cis-methylene-interrupted isomers of linoleic acid. The natural 9,12-isomer was found to be the best substrate, and the 13,16-isomer 50% as effective. The presence of calcium ions broadened the pattern of specificity. The rates of reaction of a series of polyunsaturated acids in which chain length, number of double bonds, and positions of double bonds varied were tested with crude lipoxidase in the presence of calcium ion. The substrates which exhibit high rates of reaction all possessed double bonds at the 6th and 9th carbon atoms counting from the methyl group. Thus the terminal structure is critical to the specificity of the enzyme. For enzyme reaction, the carboxyl group must not be sterically hindered, and the unsaturation at the 6th carbon atom from the methyl end of the chain must be a double bond.

Lipoxidase has been shown to attack a number of plurality unsaturated fatty acid derivatives (1), and Koch, Stern, and Ferrari (2) have shown that the crude soybean extract usually used contains several lipoxidase enzymes. Because the common assay method uses salts as substrates, most attention has been centered on the enzyme which attacks acids and less is known about triacylglyceride lipoxidase. Free acid lipoxidase requires a methylene-interrupted system of cis double bonds, and the product is a conjugated diene hydroperoxide (1). With linoleate as substrate, Hamberg and Samuelsson (3) found that the peroxide group in the product was 70% at position 13 (w6) and 30% at position 9. Dolev, Rohwedder, and Dutton (4) found the peroxide at the w6 position. These observations, plus the fact that the product of lipoxidase reaction is optically active, whereas the product from autoxidation is not (6), indicate that the enzyme attack is highly specific.

The specificity of the enzyme for positional structure of the substrate has been investigated previously by Hamberg and Samuelsson (3, 5), who observed that of the eight all-cis polyunsaturated acids tested, the seven acids which had a diene system which begins with the 6th carbon atom counted from the terminal methyl group (w6) reacted to yield w6 hydroperoxides. 8,11,14-Docosatrienoic acid (22:3w6) was not a substrate for lipoxidase.

The availability of a complete series of isomers of linoleic acid (7) in this laboratory made possible a more thorough evaluation of the substrate specificity. The rates of lipoxidase oxidation have also been studied for a long series of polyunsaturated acids in which the chain length and positions of double bonds vary. The results in part confirm and in part extend the observations of Hamberg and Samuelsson.

EXPERIMENTAL PROCEDURE

Crude lipoxidase used in preliminary studies and for studies with the polyunsaturated acids of differing chain length, number of double bonds, and double bond positions was obtained from Worthington. The solution used contained 1 mg per ml of protein which assayed 2,170 units per mg, and had 3.8 units per unit absorbance at 270 m. For study of the isomers of linoleic acid, a highly purified preparation was used. This preparation had been isolated (8) and characterized (9) in 1947 as a single protein entity capable of being crystallized. The pure enzyme had been kept under saturated ammonium sulfate solution at −20° for 20 years, and was found to retain 5% of its original activity. Therefore, this preparation, although not having the original potency, was uncontaminated by other forms of lipoxidase and other enzymes present in crude soybean extract. The solution of this enzyme used had 9.0 units per absorbance unit at 270 m or 10,240 units per mg of protein. Both enzyme preparations were dialyzed against water and filtered before use.

The isomeric octadecadienoic acids were synthesized via alkyl-lactone intermediates (7). The 10,13,16-19:3, 6, 9, 12, 15-21:4, 1 The number preceding the colon indicates the chain length of the fatty acid; the number following the colon indicates the total number of double bonds.

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Fig. 1. Substrate specificity of purified soybean lipoxidase upon positional isomers of octadecadienoic acid in which the methylene-interrupted cis, cis-diene system occurs from the 9,5- to the 14,17-positions. The abscissa is numbered with reference to the carboxyl group. Rates of reaction are plotted relative to the rate for natural linoleic acid.

5,8,11-17:3, 9,12-17:2, 10,13-19:2, and α-methylarachidonic acids were obtained through the courtesy of H. Schlenk and T. Gerson of this institute. I. Wolff of the Northern Regional Research Laboratory provided the sample of crepenynic acid. The remaining acids were prepared from natural sources or synthesized in this laboratory, or purchased from the Lipids Preparation Laboratory of The Hormel Institute. All were of known structure tested by ozonolysis, and more than 95% purity as tested by gas-liquid chromatography.

Each acid (0.1 g) was dissolved with shaking in 60 ml of ethanol and made up to 100 ml with distilled water to prepare a stock solution, which for linoleic acid is 3.56 x 10⁻³. Just prior to use, 20 ml of stock solution were diluted with 100 ml of 0.2 M borate buffer, pH 9.0, to give the substrate solution. With the isomeric acids, the incubation was carried out at 30° with 0.5 ml of substrate, 2.0 ml of oxygenated borate buffer, and 0.5 ml of purified enzyme preparation. In the study with polyunsaturated acids, the incubation was carried out with 1.0 ml of substrate, 4.0 ml of oxygenated buffer, and 65 μl of a solution of commercial lipoxidase. After the addition of enzyme, the course of the reaction was recorded versus time with a Beckman DK2 ratio recording spectrophotometer set at 234 μμ, with the appropriate blank. Details of assay and definition of units can be found in Reference 10. After preliminary study of the effect of calcium upon lipoxidase, which confirmed that it activates the enzyme (11), calcium chloride was added to the substrate solution to provide Ca⁺⁺ at the final concentration of 1 x 10⁻⁵ M. This concentration was used in all cases except in one series in which no calcium was added.

The rates of reaction during the first 3 min of incubation were calculated from the slopes of the recordings for five replicates of each substrate in the studies of the action of purified lipoxidase upon isomer 18:2 acids with or without calcium. In the studies of the action of crude enzyme upon other polyunsaturated acids, three replicates were measured for each substrate. Because a limited number of substrates could be assayed in 1 day and because lipoxidase activity of a preparation diminishes with time, natural linoleic acid was included in each day's work as a standard. All measurements of activity, based upon diene conjugation, were related to the activity upon linoleic acid, which gave the highest yield of product.

In Fig. 1 the yields of diene conjugation from the isomers of linoleic acid are displayed as a function of the positions of the first double bond in each, counting from the carboxyl group. It is apparent that linoleic acid, 9,12-18:2, is the best substrate among the isomers. The discrimination of the enzyme for double bond position is of a high order, for the neighboring isomers, 8,11- and 10,13-18:2, are oxidized to the extent of only 13 and 21%, respectively, in the absence of calcium ion. When the double bonds of the substrate approach the carboxyl group, the enzyme activity approaches zero. All isomers in which the double bonds are beyond the 9,12-positions are oxidized to some extent. The second peak of activity exhibited by the 13,16-isomer, amounting to 50% of the reaction with the natural 9,12-isomer, is noteworthy.

When calcium ions were present in the incubation medium, the activities on all isomers were increased above the controls without calcium (9). However, relative to linoleic acid itself, the same pattern of substrate specificity was elicited by the series of isomers of 18:2. The presence of calcium broadened the pattern of specificity of lipoxidase, increasing the relative rates of reaction for the 7,10-, 8,11-, 10,13-, and 11,14-isomers. That is, the discriminating capability of the enzyme, or the preference for the 9,12-isomer, was diminished.

In the second portion of the study, a number of fatty acids which differed by chain length, number of double bonds, and double bond positions were peroxidized by crude soybean lipoxidase in the presence of calcium. In Table I these substances are listed in order of decreasing rate of peroxidation. There is no apparent correlation between the positions of the double bonds in relation to the carboxyl group and the rates of peroxidation. On the other hand, all substrates which were oxidized at high rates had double bonds at the 6th and 9th carbon atoms counting from the terminal methyl group.

### Table I

**Rates of oxidation of polyunsaturated fatty acids by crude soybean lipoxidase in presence of Ca⁺⁺**

<table>
<thead>
<tr>
<th>Oxidation rate</th>
<th>Substance (Geneva numbering)*</th>
<th>Positions of double bonds counting from terminal methyl group</th>
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<tbody>
<tr>
<td>100</td>
<td>9,12-18:2</td>
<td>6 9</td>
</tr>
<tr>
<td>93.6</td>
<td>4,7,10,13,16,19-22:6</td>
<td>3 6 9 12 15 18</td>
</tr>
<tr>
<td>91.4</td>
<td>5,8,11,14,17-20:5</td>
<td>3 6 9 12 15</td>
</tr>
<tr>
<td>86.9</td>
<td>11,14-20:2</td>
<td>6 9</td>
</tr>
<tr>
<td>83.3</td>
<td>10,13,16-19:3</td>
<td>3 6 9</td>
</tr>
<tr>
<td>79.0</td>
<td>9,12,15-18:3</td>
<td>3 6 9</td>
</tr>
<tr>
<td>76.8</td>
<td>5,8,11-14-20:4</td>
<td>6 9 12 15</td>
</tr>
<tr>
<td>55.7</td>
<td>11,14,17-20:3</td>
<td>3 6 9</td>
</tr>
<tr>
<td>44.0</td>
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</tr>
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<td>3 6 9</td>
</tr>
<tr>
<td>27.6</td>
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</tr>
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<td>4 7 10</td>
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<td>3.5</td>
<td>10,13-19:2</td>
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<tr>
<td>3.8</td>
<td>Crepenynie acid</td>
<td>6 9</td>
</tr>
<tr>
<td>0</td>
<td>2-Methylarachidonic acid</td>
<td>6 9 12 15</td>
</tr>
</tbody>
</table>

*For explanation, see Footnotes 1 and 2 of text.*

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R. B. Koch and R. T. Holman, unpublished data.
from the methyl group. These acids were good substrates for lipoxidase, regardless of chain length (even or odd), number of double bonds, or terminal structure. The presence of a double bond at the ω3 position did not hinder lipoxidase activity. That the unsaturation at the ω6 carbon must be a double bond was shown by the low rate of oxidation exhibited by crepenynic acid (octadeca-9-en-12-ynoic acid). Other factors must govern the rate of reaction, for the presence of double bonds at the ω6 and ω9 positions does not guarantee a high rate of reaction. Nevertheless, all substrates with high rates of reaction possessed double bonds in these positions.

From these observations, it is apparent that the number of carbon atoms from the carboxyl group to any double bond in the substrate molecule is not correlated with the rate of reaction and is not critical to the enzyme's reaction with the substrate. On the other hand, there is a high correlation between the rate of reaction and the presence of double bonds at the 6th and 9th carbon atoms counting from the methyl group. The 6th carbon atom is that one at which the OOH group appears in the reaction product from linoleic acid (4). This carbon atom also bears a double bond in the 13,16-octadecadienoic acid (18:202) which was found to be a good substrate for lipoxidase in the study shown in Fig. 1. These observations suggest that unsaturation involving carbon 6, counting from the methyl group, is a requisite for efficient lipoxidase action. It should be noted that the 13,16-isomer also exhibited maxima in the profiles of acyl transferase activities of liver microsomes with the CoA esters of the same series of isomeric 18:2 acids (12). This suggests that the positions of double bonds in this isomer confer some unique property upon that fatty acid.

Of the approximately 881 amino acid residues in the protein molecule, 307 are amino acids whose side chains are nonpolar: alanine, isoleucine, leucine, phenylalanine, and valine (13). Thus, the enzyme could have islands or clefts of nonpolar surface offering attraction to the long hydrocarbon chain of the substrate. The enzyme surface must have some geometric characteristic which orients the substrate so that the attack by oxygen is at the 6th carbon atom from the methyl group, if this carbon is unsaturated. A free, unhindered carboxyl group of the substrate is required because α-methylarachidonic acid was found to have zero rate of oxidation by crude lipoxidase, with or without added calcium. Arachidonic acid itself is a very good substrate for lipoxidase, but the presence of an α-methyl group sterically hindered the enzyme from oxidizing the derivative whose double bond structure was unaltered. The activation of lipoxidase by calcium suggests that a bound calcium atom may be the site of the enzyme to which the carboxyl group of the substrate is attracted.

REFERENCES

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