CONJUGATED DIHYDROPEROXYOCTADECATRIENOIC FATTY ACIDS FORMED UPON DOUBLE DIOXYGENATION OF α-LINOLENIC ACID BY LIPOXYGENASE-2 FROM SOYBEANS

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Abstract In the course of studies on the properties of soybean lipoxygenase-2, it was found that this enzyme is capable of introducing 2 molecules of dioxygen in a substrate fatty acid containing a 1,4,7-octatrienoic moiety. Using α-linolenic acid as a substrate, it was observed that the dioxygenation takes place at the 9- and 16-positions under formation of a conjugated triene system. The products were identified by HPLC, UV-spectroscopy and GC-MS.

1. INTRODUCTION

Lipoxygenase [EC 1.13.11.12] catalyses the formation of hydroperoxides from unsaturated fatty acids containing a 1,4 pentadiene system and molecular oxygen. In soybeans, a number of isoenzymes have been found. Lipoxygenase-1 has its pH-optimum at 9.0 and produces specifically 13S-hydroperoxy-9Z-11E-octadecadienoic acid from linoleic acid. A second type of enzyme, designated lipoxygenase-2, shows a very low activity at pH 9.0, unless its substrate inhibition is counteracted by the addition of Ca^{2+} or by lowering the substrate concentration to less than 100μM[1].

With linoleic acid as the substrate, under these conditions a mixture of hydroperoxides is produced which contains predominantly 9R-hydroperoxy-10E,12Z-octadecadienoic acid. The enzyme exhibits a considerable activity at pH 6.6 even at high substrate concentrations. However, the stereoselectivity in the introduction of oxygen is less pronounced under these conditions.

In recent years, increasing attention has been paid to enzymic oxygenations of fatty acids containing more than two double bonds. For example, with arachidonic acid as the substrate, oxygen is introduced by lipoxygenase-1 at C-15 to yield 15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HPETE). The latter can in turn be converted into a mixture of dihydroperoxy-compounds: 8,15-dihydroperoxy-5Z,9E,11Z,13E-eicosatetraenoic acid (60%) and 5,15-dihydroperoxy-6E,8Z,11Z,13E-eicosatetraenoic acid (40%)[2]. Upon incubation of arachidonic acid at pH 6.6 with soybean lipoxygenase-2, the formation of prostaglandin-like material has been reported[3]. α-Linolenic acid methyl ester yielded at pH 6.0 mainly monohydroperoxides, but also hydroperoxyendoperoxides[4].

In animal systems, a variety of conjugated trienoic acids has been found as a result of lipoxygenase-catalysed conversions of arachidonic acid[5]. One might wonder whether conjugated trienoic acids could be formed from polyunsaturated fatty acids from plant origin by the action of plant lipoxygenases. In the present study the double dioxygenation of α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid) by soybean lipoxygenase-2 was investigated. Obviously, lipoxygenase-1 cannot perform a double dioxygenation of this substrate because of the imperative hydroperoxidation at C-13 by this enzyme[6].

2. MATERIALS AND METHODS

2.1 Enzyme Purification and Assay

Lipoxygenase-2 was purified from soybean (Glycine max (L.) Merr. var. Williams) according to Slappendel[7]. The specific activity was 12µMol O_2/min.mg protein, as measured polarographically with a Clark electrode mounted in a Gilson 5/6 Oxygraph thermostated at 25°C. As a substrate, 1.8mM ammonium linoleate in air-saturated 0.1M sodium borate buffer, pH 9.0 was used. Substrate inhibition of lipoxygenase-2 was counteracted by the addition of $CaCl_2$ to a final concentration of 2mM.

2.2 Incubation

α-Linolenic acid (100µM) was incubated in 500ml 0.1M sodium borate buffer (pH 9.0) at 0°C with 30 enzyme units. The reaction was monitored by taking spectra from 350-220 nm on a Cary 118C. When the reaction had stopped, due to enzyme inactivation, another 30 units were added. This procedure was repeated until no further conversion of the substrate could be achieved (approx. 3 hr). Then the incubation mixture was acidified to pH 2.5. After reduction of the hydroperoxy- and oxo-groups in the products by $NaBH_4$ (1g under stirring at room temperature for 1 hr) the mixture was acidified to pH 2.5 and extracted with diethyl ether, dried and evaporated to dryness. Finally, the products were esterified with diazomethane.

2.3 HPLC-separation

The products were analysed on a Partisil-5 column (4.6mm x 25cm) with hexane isopropanol (95:5, v/v) as the eluent. A Perkin-Elmer Series 1 LC pump with a Perkin-Elmer LC85 Spectrophotometric Detector were used. The column effluent

was monitored at 254nm and UV-spectra of the peaks were recorded. Preparative separation of the double dioxygenation products was done on a Partisil-5 column (9mm x 25cm) with the same eluent.

2.4 Further Derivatisation and GC/MS

GC/MS was performed on products, in which the hydroxy-groups had been trimethylsilylated as previously described[2] on a Kratos MS 80 with a 3% OV 101 column.

3. RESULTS AND DISCUSSION

During the incubation of α-linolenic acid with lipoxygenase-2, an increase in absorbance in the conjugated triene region was observed concommitant with that in the conjugated diene region. This suggests that α-linolenic acid and its 9-hydroperoxide are almost equally suitable as substrates. This feature seems to be different from the double dioxygenation of arachidonic acid by lipoxygenase-1, which proceeds in two distinct steps[8].

In Fig. 1, the HPLC-separation of the esterified reduced reaction products is shown. The large peaks in fraction 2 had the retention times and the absorption spectra (λ_{max} = 234nm) typical of hydroxydienes. Fractions 4-6 showed clear conjugated triene spectra (Fig. 2). After trimethylsilylation GC/MS-analysis of fraction 5 demonstrated that this compound had oxygen inserted on the 9- and 16-positions (Fig. 3).

Because of the limited stereospecificity[1] of the lipoxygenase-2 and the introduction of two chiral centres with the double dioxygenation, the formation of diastereomers of 9,16-dihydroxy-10E,12Z,14E-octadecadienoic acid is

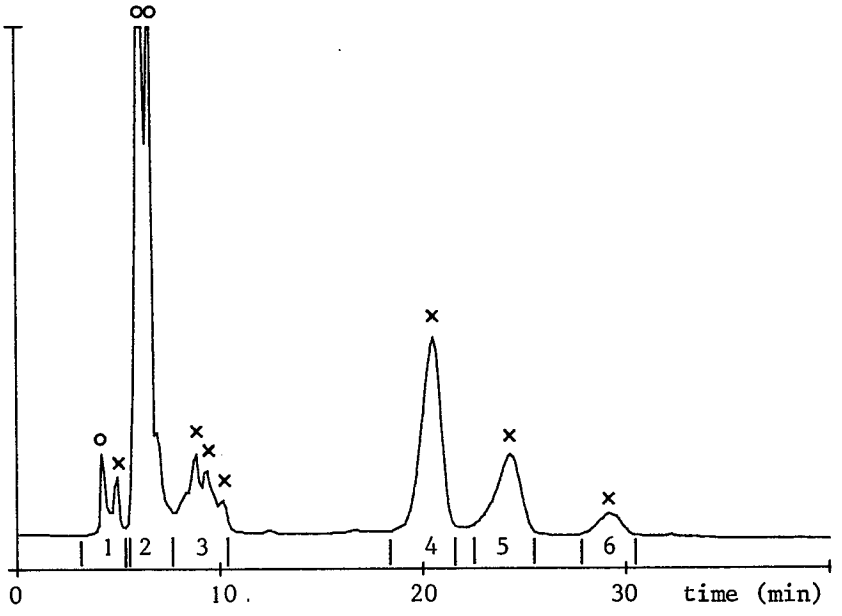

FIGURE 1 HPLC-separation of the incubation products. Detection at 254nm. o and x: absorption maximum at 234nm and 266nm, respectively.

anticipated. Assuming that the double bond configuration is E,Z,E in all products, two conjugated triene peaks can occur. However, we observe three major conjugated triene peaks with the retention times of dihydroxy compounds. The most probable explanation is the occurrence of a set of geometrical isomers, in which all double bonds have the E-configuration.

In conclusion, the formation of double dioxygenation conjugated triene products from a plant lipid by a plant lipoxygenase is clearly demonstrated. In view of the wide range of physiological effects exerted by the conjugated trienes (leukotrienes) derived from arachidonic acid in

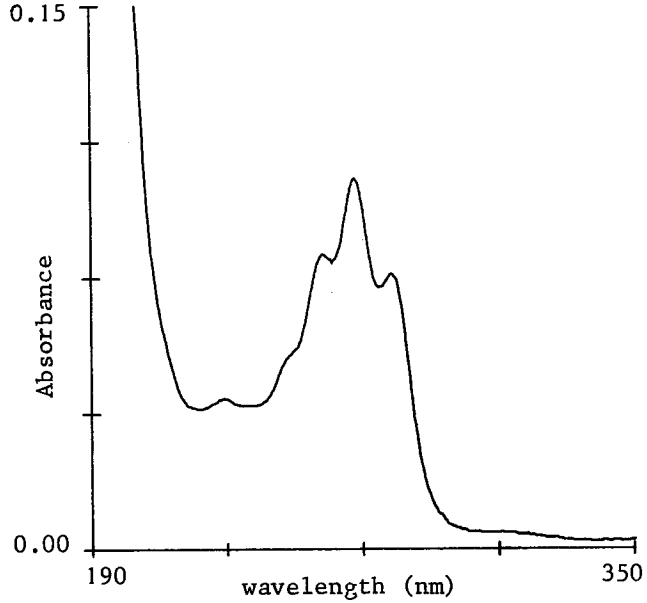

FIGURE 2 UV-spectrum of fraction 5 obtained from preparative HPLC-separation (see Fig. 1). Solvent: hexane : isopropanol (95 : 5, v/v).

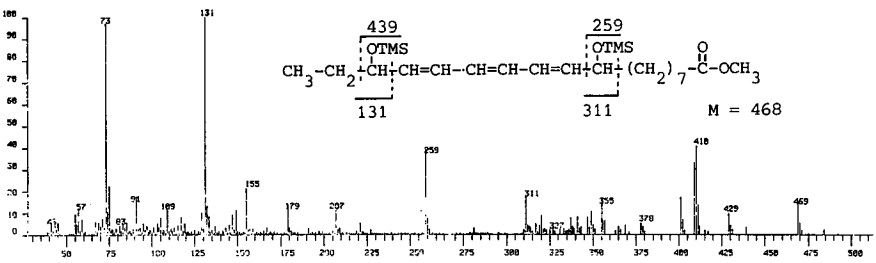

FIGURE 3 Mass spectrum of the trimethylsilylated fraction 5.

the animal kingdom[5], it is tempting to postulate comparable significance for the new trienoic compounds, described in this paper, in the plant kingdom. It is conceivable that the phytotrienes, as we coin them, could play a role as chemical messengers in the areas where plant lipoxygenase involvement has been claimed, i.e. in germination and wound healing[9].

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