Purification and Characterization of Tomato Leaf (Lycopersicon esculentum Mill.) Hydroperoxide Lyase

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INTRODUCTION

Fatty acid hydroperoxides are key components formed by the action of lipoxygenase (EC 1.13.11.12) on 1(Z),4-(Z)-pentadiene-containing fatty acids. Depending on the origin of the lipoxygenase and on the reaction conditions, variable amounts of 13- and/or 9-hydroperoxides are formed. The hydroperoxides can be further degraded in a variety of products involved in essential physiological roles in plants (jasmonic acid, traumatin) or responsible for the characteristic green notes of plants and fruits (C6 or C9 aldehydes and alcohols). Hydroperoxide lyase is an enzyme that catalyzes the cleavage of fatty acid hydroperoxides into aldehydes and ω-oxo acids. The break takes place between the carbon that contains the hydroperoxide group and the proximate ethylenic carbon. Hydroperoxide lyase was first suspected in banana (Tressl and Drawert, 1973), and its presence was later demonstrated in various plant leaves and fruits (Hatanaka et al., 1986). Hydroperoxide lyases can be classified in two categories on the basis of their substrate specificity: the 13-HPOOH lyase and the 9-HPOOH lyase. The first type degrades 13-LnOOH into 3(Z)-hexenal and 12-oxo-9(Z)-dodecenio acid and 13-LaOOH into hexanal and 12-oxo-9(Z)-dodecenio acid; the second type degrades 9-LnOOH into 3(Z),6(Z)-nonadienial and 9-oxononanoic acid and 9-LaOOH into 3(Z)-nonenal and 9-oxononanoic acid. The first type has been described in watermelon seedlings (Vick and Phillips, 1976), and soybean seedlings (Gardner et al., 1991) exhibits both activities. In cucumber cotyledons (Matsui et al., 1989), it was demonstrated that the HPOOH lyase consisted of two isomers: one specific for the 9-isomer and the other specific for the 13-isomer. HPOOH lyase is difficult to study because it is a membrane-bound enzyme and because it is present in small amounts in plant tissues. It was only purified to apparent homogeneity in tea leaves (Matsui et al., 1991) and in green bell pepper fruits (Shibata et al., 1995a), in which it was shown to be a heme protein (Shibata et al., 1995b). We have reinvestigated the extraction and the purification of the HPOOH lyase from tomato leaves, resulting in a quite simple procedure. The pl and molecular mass of the enzyme and of its subunits were determined; particular attention was paid to the specificity of the enzyme for its substrate. The inhibition of the enzyme by fatty acid hydroperoxides was studied systematically: the enzyme was preincubated with 9- and 13-OOH from α-linolenic acid, γ-linolenic acid, and linoleic acid. The inhibitory effect of 13-LnOOH was also checked after reduction to the corresponding alcohol and after esterification with methanol. The aldehydes, products of the reaction, and the corresponding alcohols were also used for the inhibitory assays.

This paper contributes to a better knowledge of HPOOH lyase, which has been poorly studied in spite of its physiological importance. The enzyme is essential for fruit aroma synthesis and has an application in the aroma industry for the production of natural green note compounds. The reaction products (aldehydes, oxo acids) are implicated in plant defense against pathogens and in wound healing.

MATERIALS AND METHODS

Material. HPOOH lyase was purified from tomato leaves (Lycopersicon esculentum Mill. var. Bonset) cultivated in the Institut Supérieur Industriel (Grand-Manil, Belgium). The potato tubers originated from the local market. EDTA, diethiothreitol, Triton X-100, PEG 6000, 2-mercaptoethanol, NADH,
linoleic acid (purity by GC > 99%), α-linolenic acid (purity by GC > 98%), γ-linolenic acid (purity by GC > 99%), yeast alcohol dehydrogenase, and soybean lipoxigenase (type I-S) were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

**Methods.** Enzyme Purification. All of the experiments were conducted at 4 °C. Tomato leaves (170 g) were extracted directly in a Waring blender (three times, 1 min) with 200 mL of sodium acetate buffer (50 mM, pH 5.5) containing 3 mM EDTA, 3 mM dithiothreitol, and 0.5% (v/v) Triton X-100. The extract was centrifuged (15 min, 12000 × g) and the supernatant was magnetically agitated (90 min) and centrifuged again (20 min, 26000 × g). PEG 6000 (7%, w/v) was added slowly to the supernatant. After 30 min of agitation, the extract was centrifuged (20 min, 26000 × g). PEG was added to the supernatant to obtain a final concentration of 23% (w/v). The solution was again agitated for 30 min. The extract was centrifuged (15 min, 12000 × g), and the residue was solubilized in 15 mL of Tris-HCl buffer (20 mM, pH 9) containing 0.5% (v/v) Triton X-100. PEG was added to obtain a final concentration of 3% (w/v), and the extract was agitated for 30 min and centrifuged (12 min, 12000 × g). Tris-HCl buffer (5 mL) was used to solubilize the residue. 2-Mercaptoethanol was added to the extract to obtain a final concentration of 0.1 mM.

The extract was loaded on a DEAE-Sepharose CL 6B column (Pharmacia, Uppsala, Sweden) column (1.5 × 25 cm) equilibrated with 20 mL of Tris-HCl buffer (pH 8.5) containing 0.5% (v/v) Triton X-100 and 0.1 mM 2-mercaptoethanol. The column was calibrated with the gelfiltration standard kit from Bio-Rad (Richmond, CA). The standards were the high molecular weight calibration kits from Bio-Rad and Pharmacia.

**Enzyme Puriﬁcation.** 13-La OOH, 13-Ln OOH, and 13-γ-Ln OOH were used to solubilize the residue. 2-Mercaptoethanol was added to the extract to obtain a final concentration of 0.1 mM. The extract was centrifuged (15 min, 12000 × g) and the supernatant was magnetically agitated (90 min) and centrifuged again (20 min, 26000 × g). PEG 6000 (7%, w/v) was added slowly to the supernatant. After 30 min of agitation, the extract was centrifuged (20 min, 26000 × g). PEG was added to the supernatant to obtain a final concentration of 23% (w/v). The solution was again agitated for 30 min. The extract was centrifuged (15 min, 12000 × g), and the residue was solubilized in 15 mL of Tris-HCl buffer (20 mM, pH 9) containing 0.5% (v/v) Triton X-100. PEG was added to obtain a final concentration of 3% (w/v), and the extract was agitated for 30 min and centrifuged (12 min, 12000 × g). Tris-HCl buffer (5 mL) was used to solubilize the residue. 2-Mercaptoethanol was added to the extract to obtain a final concentration of 0.1 mM.

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Native-PAGE was performed on a Mini-Protean II cell (Bio-Rad, Hercules, CA). SDS-PAGE was carried out on the same kind of gel as the native-PAGE using the high-range molecular mass from Bio-Rad as standards.

**RESULTS AND DISCUSSION**

**Enzyme Purification.** The use of Triton X-100 was very efficient to solubilize the enzyme from the membranes. The concentration of 0.5% was the optimal compromise between solubilization and enzyme inhibition. The fractioned precipitation with PEG 6000 between 7% and 23% (w/v) was a very simple first step of purification, allowing an increase in specific activity (aldehydes) by means of n-hexane extraction and GC determination. For this purpose, (2–x) mL of sodium phosphate buffer (0.1 M, pH 6.7), 10 μL of 13-Ln OOH in methanol (12 mM), and x μL of enzyme extract were mixed and the reaction was conducted 5 min at 25 °C. The reaction medium was saturated in (NH₄)₂SO₄ and extracted with 2 x 2 mL of n-hexane. 1-Heptanol (internal standard) was added, and the hexanic extract was analyzed by GC (Hewlett-Packard 5880) on a Carbowax column (30 m, 0.025, 0.1 mm d.). The oven temperature was held at 60 °C for 1 min, increased to 90 °C at 5 °C/min, and then increased to 220 °C at 30 °C/min.

**Substrate Synthesis and Characterization.** For 13-La OOH, 13-Ln OOH, and 13-γ-Ln OOH synthesis, respectively, 10 mg of linoleic acid, α-linolenic acid, or γ-linolenic acid and 4 mg of commercial soybean lipoxigenase were added to 100 mL of oxygenated borate buffer (0.2 M, pH 9). The reaction was carried out at 2 °C for 15 min under a constant flow of oxygen. The 9-hydroperoxides (9-La OOH, 9-Ln OOH, and 9-γ-Ln OOH) were produced using lipoxigenase extracted from potato tubers (according to the method of Galliard and Phillips (1971)). The reaction was carried out in 100 mL of oxygenated phosphate buffer (50 mM, pH 6.7) at 25 °C during 15 min.

**Hydroperoxides Extraction.** The pH values of the reaction media were adjusted to 3 with N HCl, and the reaction products were extracted on C₁₈ cartridges (C₁₈ Chromabond of 500 mg from Macherey-Nagel, Düren, Germany) according to the method of Sanz et al. (1990). 13-La OOH, 13-Ln OOH, and 13-γ-Ln OOH were purified by HPLC according to the method of Sanz et al. (1993).

**Hydroperoxide Analysis.** The positional and geometrical isomers of hydroperoxides were analyzed by HPLC after reduction with NaBH₄ and methylation with diazomethane according to the method of Sanz et al. (1993).

**Enzymatic Activity Characterization.** Substrate specificity was determined with the 234 nm test in triplicate with the following substrates: 13-Ln OOH, 9-La OOH, 9-Ln OOH, 13-La OOH, 13-γ-Ln OOH, and 9-γ-Ln OOH. The optimal temperature was determined using the 234 nm test in triplicate with 13-Ln OOH as substrate; the range of temperature tested was from 5 to 70 °C in steps of 5 °C. The optimal pH was determined in the same conditions (30 °C), using 0.1 M sodium acetate buffer for the pH range 3–4, 0.1 M sodium phosphate buffer for pH 5–8, and sodium borate buffer for pH 9–10.

**Inhibition Tests.** Kᵣ and V_max were determined in triplicate in the conditions used for the test at 234 nm. The enzyme extract was used at increasing dilutions as the substrate concentration cannot be increased because of the limitations in absorbance.

**Enzyme inhibition experiments were carried out after prunacination of the enzyme with various compounds.** Sodium phosphate buffer (0.1 M, pH 6.7, 2.99–x mL) is mixed with x mL of enzyme extract and 10 μL of a solution of the compound to be tested at the desired concentration. After 10 min, 10 μL of 13-Ln OOH (0.12 mM) was added, and the activity was determined as previously described for the test at 234 nm.

In the same way, the enzyme was preincubated with 13-Ln OOH as described above and dialyzed for 2 days against distilled water (the absence of hydroperoxide was checked by following the absorbance at 234 nm). The activity of the enzyme was then determined using the test at 234 nm. A blank was realized exactly in the same conditions but without prunacination of the enzyme with its substrate.

The positional and geometrical isomers of hydroperoxides were analyzed by HPLC after reduction with NaBH₄ and methylation with diazomethane according to the method of Sanz et al. (1993).
Table 1. Purification of HPOOH Lyase from Tomato Leaves

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg of protein)</th>
<th>Yield (%)</th>
<th>Purification Factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>425</td>
<td>530</td>
<td>1.2</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Supernatant after addition of PEG 7%</td>
<td>350</td>
<td>430</td>
<td>2.9</td>
<td>81</td>
<td>2.4</td>
</tr>
<tr>
<td>Residue after second addition of PEG 23%</td>
<td>55</td>
<td>250</td>
<td>4.5</td>
<td>47</td>
<td>3.7</td>
</tr>
<tr>
<td>Fraction III DEAE Sepharose CL-6B</td>
<td>1.20</td>
<td>130</td>
<td>108.3</td>
<td>25</td>
<td>90.2</td>
</tr>
<tr>
<td>Extract after three cycles of ultrafiltration</td>
<td>0.55</td>
<td>79</td>
<td>143.6</td>
<td>15</td>
<td>120.0</td>
</tr>
</tbody>
</table>

* After centrifugation and solubilization.

Figure 1. DEAE-Sepharose CL 6B elution profile.

![Image](54x470 to 294x651)

**Figure 2.** SDS-PAGE profile: (lane 1) residue after the second precipitation with PEG 23%; (lane 2) DEAE-Sepharose CL 6B active fraction; (lane 3) after one cycle of ultrafiltration; (lane 4) after three cycles of ultrafiltration; (lane 5) molecular mass standards.

of 3.7 and a recovery percentage of 47% (Table 1). The extract obtained here exhibited a great tendency to aggregate during chromatographic procedures. The conditions described under Materials and Methods are critical to avoid the elution of the enzyme in the void volume of the DEAE-Sepharose CL-6B column. Indeed, the use of Triton X-100 alone at whatever concentrations is not efficient to allow enzyme to interact with the column. The use of 2-mercaptoethanol at 0.1 mM combined with the Triton X-100 gave the best results (Figure 1). A 24-fold increase in specific activity was obtained for this purification step. The electrophoretic pattern shows that a few contaminant proteins with a molecular mass <75 000 Da were still present in the extract (Figure 2). As the HPOOH lyases are known to be high molecular mass enzymes, we decided to use an ultrafiltration membrane with a cutoff of 100 000 Da to remove the contaminants. Three cycles of ultrafiltration were needed to obtain a single band on the electrophoretic pattern. For all purification procedures, the activity was determined by the decrease in absorbance at 234 nm. In the DEAE step, the spectrophotometric activity determination was also correlated with the aldehyde synthesis by the enzyme (Figure 1). The combined test at 340 nm was used to measure the activity on the crude extract and on the final extract. In the crude extract, 77% of the hydroperoxide degradation activity came from HPOOH lyase; the rest can result from hydroperoxide dehydrase activity or from another hydroperoxide metabolizing enzyme activity. In the final extract, all of the hydroperoxide metabolizing activity was attributed to HPOOH lyase.

**Enzyme Characteristics.** The extract obtained previously was submitted to SDS–PAGE, revealing a single band at 73 000 Da. The native–PAGE also exhibited a single band with a molecular mass between 200 000 Da (catalase) and 232 000 Da (myosine). The accurate determination of molecular mass was difficult because native–PAGE gives quite broad bands. Gel filtration on Superdex 200, after calibration with standards, gave a molecular mass for the native protein of 216 000 Da. The enzyme is then supposed to be a trimer of three 73 000 Da subunits for a total molecular mass of ~216 000 Da. The molecular mass of the enzyme has been determined to be 220 000 Da in spinach leaves (Vick and Zimmerman, 1987), between 240 000 and 260 000 Da in soybean cotyledons (Olias et al., 1990), and ~200 000 Da in tomato fruits (Schreier and Lorenz, 1982). The enzyme is thought to be a tetramer of four 62 000 Da subunits in soybean cotyledons, while recently Shibata et al. (1995a) demonstrated that the enzyme was a trimer of 55 000 Da subunits in green bell pepper fruit. In tea leaves, subunits have also a molecular mass between 53 000 and 55 000 Da (Matsui et al., 1991).

The pI of the enzyme determined by isoelectrofocalization in the presence of standards revealed that the enzyme has a pI of 4.9. A single band was present on the electrophoretic pattern, meaning that the enzyme is presumed to exist as a single isoform in tomato leaves. In tomato fruits, Schreier and Lorenz (1982) have demonstrated that the pI of HPOOH lyase was between 5.8 and 6.1. The HPOOH lyase extracted from tomato leaves is quite different from the other enzymes described in the literature, not only because of the molecular mass of the native form of the enzyme, which is very common, but also because of the size of the subunits (73 000 Da) and the pI value.

**Enzyme Activity Characteristics.** The optimal pH for tomato leaf HPOOH lyase is 7; the activity is high (90% ± 4% of the maximum activity) at pH between 6 and 8 but decreases to 60% ± 3% at pH 4 and to 70% ± 5% at pH 9, meaning that the enzyme has a quite broad range of working pH. Optimal pH values described for HPOOH lyase from other origins range from 5.5 in green bell pepper fruit (Shibata et al., 1995a) to 8 in cucumber fruits (Matsui et al., 1989), while the one extracted from pear fruits (Kim and Grosch, 1981), tea leaves (Matsui et al., 1991), and soybean cotyledons (Olias et al., 1990) work at neutral pH. Optimal pH
Table 2. Analysis of the Hydroperoxides Synthesized Using Soybean or Potato Lipoxigenase

<table>
<thead>
<tr>
<th>regiosomer proportion, 13-OOH/9-OOH</th>
<th>stereoisomer proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>13-La OOH</td>
<td>98/2</td>
</tr>
<tr>
<td>13-Ln OOH</td>
<td>9/1</td>
</tr>
<tr>
<td>13-γ-Ln OOH</td>
<td>98/2</td>
</tr>
<tr>
<td>9-La OOH</td>
<td>96/4</td>
</tr>
<tr>
<td>9-Ln OOH</td>
<td>98/2</td>
</tr>
<tr>
<td>9-γ-Ln OOH</td>
<td>98/2</td>
</tr>
</tbody>
</table>

described for HPOOH lyase from tomato fruits is 5.5 according to Schreier and Lorenz (1982). Riley et al. (1996) observed a high enzymatic activity between pH 6 and 8 for HPOOH lyase of tomato fruits; this last result is very similar to our observations.

The optimal temperature of the enzyme is 30 °C. At 20 °C, the activity is 63% ± 4% of that at 30 °C, while at 40 °C it is still 90% ± 5% and at 50 °C, 77% ± 3%.

The specificity of the enzyme against various hydroperoxides was determined. The analysis of the hydroperoxides produced as described under Materials and Methods is presented in Table 2. The 13-hydroperoxides were used without further purification, while the 9-hydroperoxides required HPLC purification because of the lack of specificity of potato lipoxigenase. The results obtained for the study of the enzyme specificity against the six different hydroperoxides revealed that the enzyme has no activity against 9-La OOH, 9-Ln OOH, 9-γ-Ln OOH, and 13-γ-Ln OOH. The relative activities against 13-Ln OOH and 13-La OOH are, respectively, 100% and 9.8%. This specificity is also encountered in tea leaf HPOOH lyase, for which the relative activities against the same substrates are 100% and 10.9% (Matsui et al., 1991), although, in this last case, the authors also determined a small activity against 13-γ-Ln OOH (0.8%). Similar results have been described in green bell pepper fruits by Shibata et al. (1995a). In soybean seedlings, Olias et al. (1990) demonstrated that the HPOOH lyase was more specific for the 13-La OOH than for 13-Ln OOH. It seems that the specificity of the enzyme is a function of the fatty acid composition in vivo; α-linolenic acid is preponderant in leaves and in green bell pepper fruits, while linoleic acid is very abundant in seedlings. The K_m values determined for tomato leaf HPOOH lyase are 60 and 34 µM for 13-Ln OOH and 13-La OOH, respectively; those K_m values are between those determined in green bell pepper fruits (Shibata et al., 1995a) and soybean seedlings (Olias et al., 1990). The V_max values are 16 mM/min and 9.4 µM/min, respectively, corresponding to K_cat values of 154 880 mol of 13-Ln OOH/min·mol of enzyme and 65 800 mol of 13-La OOH/min·mol of enzyme. The kinetic parameters of HPOOH lyase are quite ambiguous because the K_m values show a greater affinity for 13-La OOH, while V_max and K_cat indicate the contrary. This compartment can be explained by a difference in the kinetic constants: K_2 (constant of dissociation of the enzyme–product complex) must be higher for 13-Ln OOH than for 13-La OOH, explaining the values of V_max. On the other hand, the ratio K_2/K_1 (constant of formation of the enzyme–substrate complex) must be higher for 13-La OOH, explaining the lower K_m value. Finally, the catalytic efficiency constant (K_cat/K_m), which is frequently used to determine the affinity of an enzyme for a particular substrate, clearly demonstrates the greater affinity for 13-Ln OOH: K_cat/K_m is 2.58 × 10^9 min⁻¹·mol⁻¹ of enzyme⁻¹ for 13-Ln OOH and 1.85 × 10^8 min⁻¹·mol⁻¹ of enzyme⁻¹ for 13-La OOH.

Table 3. Residual Activity after Preincubation of HPOOH Lyase with Different Hydroperoxides

<table>
<thead>
<tr>
<th>concn (µM)</th>
<th>residual relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>100 ± 2.5</td>
</tr>
<tr>
<td>13-Ln OOH</td>
<td>40 74 ± 2.0</td>
</tr>
<tr>
<td>13-Ln OOH</td>
<td>90 44 ± 2.2</td>
</tr>
<tr>
<td>13-La OOH</td>
<td>40 70 ± 4.1</td>
</tr>
<tr>
<td>13-γ-Ln OOH</td>
<td>49 55 ± 3.3</td>
</tr>
<tr>
<td>9-Ln OOH</td>
<td>40 44 ± 3.1</td>
</tr>
<tr>
<td>9-La OOH</td>
<td>40 45 ± 2.8</td>
</tr>
<tr>
<td>9-γ-Ln OOH</td>
<td>40 47 ± 3.3</td>
</tr>
<tr>
<td>13-Ln OOH^a</td>
<td>40 71 ± 2.6</td>
</tr>
<tr>
<td>13-Me-Ln OOH^b</td>
<td>40 90 ± 1.8</td>
</tr>
</tbody>
</table>

^a 13-Ln OOH reduced to the corresponding alcohol by NaBH₄.
^b 13-Ln OOH esterified using diazomethane.

Table 3 shows the results obtained for the inhibition of HPOOH lyase after preincubation with the different hydroperoxides or hydroperoxide-derived products. The inhibitory effect obtained with 13-Ln OOH or with 13-La OOH is less important than the one obtained with the four other hydroperoxides, which are not substrates for HPOOH lyase. A possible explanation is that part of the 13-Ln OOH or 13-La OOH is metabolized by the enzyme during the preincubation period and the enzyme is then in contact with a concentration in hydroperoxide lower than in the case of the other hydroperoxides which are not transformed. Actually, when HPOOH lyase is preincubated with 13-Ln OOH at a concentration of 40 µM, the hydroperoxides are partially degraded, and the concentration finally stabilized at 19 µM in 5 min. When the initial concentration is 90 µM, the final concentration decreased to 54 µM. Regarding those results, all of the hydroperoxides potentially or not transformable by the enzyme seem to have the same inhibitory effect on the enzyme for a given concentration. When 13-Ln OOH is transformed in the corresponding alcohol by reduction with NaBH₄, the 13-hydroxide obtained is not a substrate for the enzyme. It inhibits the enzymatic activity but to a lesser extent than the hydroperoxides. Matsui et al. (1992) obtained also an inhibitory effect with 13-hydroxide on HPOOH lyase from tea leaf, but the residual activity was 90% of the initial one, while at the same concentration the 13-La OOH resulted in a loss of relative activity of 75%. When 13-Ln OOH was esterified using diazomethane, the inhibitory effect was very weak. The inhibitory effect of fatty acid hydroperoxides on HPOOH lyase is not specific because the same effect is obtained with hydroperoxides from linoleic acid, α-linolenic acid, or γ-linolenic acid. The position of the function in the molecule is not critical, as there is no difference between 9-OOH and 13-OOH. The hydroperoxide function itself is implicated, but the polarity of the molecule also influences the mechanism. When HPOOH lyase is dialyzed against distilled water for 2 days, the residual activity is 86% ± 4%. When the enzyme is preincubated with 13-Ln OOH at a concentration of 40 or 90 µM before the dialysis, the residual activities are, respectively, 63% ± 3% and 41% ± 3%. This means that the inhibition is irreversible because the elimination of the hydroperoxides of the medium could not restore the activity.

We have also checked the effect of the aldehydes issuing from the reaction and their corresponding alcohols on the enzymatic activity. Preincubation with (E)-2-hexenal, (E)-2-hexenol, (Z)-3-hexenal, and (Z)-3-hexenol at a concentration of 0.1 mM revealed no inhibitory effect.
ABBREVIATIONS USED

HPOOH lyase, hydroperoxide lyase; 9-La OOH, 9-hydroperoxide of linoleic acid; 9-Ln OOH, 9-hydroperoxide of α-linolenic acid; 13-La OOH, 13-hydroperoxide of linoleic acid; 13-Ln OOH, 13-hydroperoxide of α-linolenic acid; 9-γ Ln OOH, 9-hydroperoxide of γ-linolenic acid; 13-γ Ln OOH, 13-hydroperoxide of γ-linolenic acid.

LITERATURE CITED


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