# Site-specific cleavage of double-strand DNA by hydroperoxide of linoleic acid

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The breakage of double-strand (ds) DNA by 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (LAHPO) was investigated by agarose gel electrophoresis of supercoiled pBR322 DNA and the site of cleavage on the DNA molecule was determined by the method of DNA sequence analysis using 3'-end and 5'-end-labeled DNA fragments as substrates. LAHPO caused cleavage at the position of guanine nucleotide in dsDNA. LAHPO caused dsDNA breaks at specific sites, but linoleic acid (LA) and 13-L-hydoxy-*cis*-9,*trans*-11-octadecadienoic acid (LAHO) have no such effects on dsDNA. The active oxygen atom of the hydroperoxy group of LAHPO was perhaps responsible for the site-specific cleavage of dsDNA.

*Lipid hydroperoxide* *Site-specific DNA cleavage* *Hydroperoxylinoleic acid* *Unsaturated fatty acid* *Lipoxygenase*

## 1. INTRODUCTION

Some hydroperoxides of unsaturated fatty acids are important intermediates of prostaglandins [1,2], leukotrienes [3–5] and other fatty acid metabolites [6,7]. On the other hand, the hydroperoxides which are formed through enzymatic and/or non-enzymatic reaction [8,9] have detrimental effects on living cells. The peroxidation of unsaturated fatty acids of cells produces many reactive species such as free radicals, hydroperoxides and carbonyl compounds, which may cause damage to proteins [8], membrane structures and cellular organization [10]. A number of biological oxidations generate the superoxide radical ($O_2^{\cdot -}$) which is cytotoxic, and in turn can react with $H_2O_2$ to produce singlet oxygen and hydroxy radicals ($OH^{\cdot}$) which act as highly potent oxidants [11,12]. The toxicity produced by lipid peroxidation has been suggested as a major cause of cancer, heart disease and ageing [13,14].

Recent investigations with bleomycin [15] and neocarzinostatin [16] suggest that active oxygen interacts with DNA and causes cleavage of DNA strands. However, the interaction of DNA with lipid peroxides has not been investigated. We examined here the effect of fatty acid hydroperoxide on dsDNA as a model, using LAHPO (fig.1) which was enzymatically prepared and purified before use.

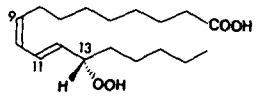


Fig.1. Structure of 13-$L_s$-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (LAHPO).

## 2. MATERIALS AND METHODS

### 2.1. Materials

Restriction endonuclease, *E.coli* alkaline phosphatase and T4 polynucleotide kinase were from Takara Shuzo (Kyoto, Japan). The Klenow frag-

*Abbreviations:* LAHPO, 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; LAHO, 13-L-hydroxy-*cis*-9, *trans*-11-octadecadienoic acid; LA, linoleic acid; dsDNA, double-strand DNA

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ment of DNA polymerase I of *E.coli* was from Bethesda Research Laboratories. [$\alpha$-$^{32}$P]dCTP and [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) used for labeling were from Amersham. Soybean lipoxygenase I (type V) was from Sigma. LA (Wako, Osaka) was further purified by dry column chromatography with silica gel (Woelm Pharma, FRG). All reagents were analytical grade, unless otherwise stated.

### 2.2. Preparation of LAHPO

LAHPO was enzymatically prepared with soybean lipoxygenase as in [17]. The structure of LAHPO was confirmed by high-performance liquid chromatography (HPLC) [18], UV, IR and NMR spectra [19]. The LAHPO obtained was single spot on silica gel thin-layer chromatography (TLC). Typical composition of LAHPO was shown in [18]. The amount of hydroperoxide was calculated from UV absorption at 234 nm ($\epsilon$ = 27 400). LAHO was prepared by reduction of LAHPO with $NaBH_4$.

### 2.3. Preparation of plasmids

The plasmid was isolated from cleared lysates and purified further by CsCl/ethidium bromide gradient centrifugation [20]. The plasmids used, pSS605 and pSS634, are cloned *Drosophila melanogaster* DNA fragments into pUC9 [21].

### 2.4. Standard reaction condition with LAHPO

The reaction mixture contained 10 mM Tris–HCl buffer (pH 7.6), supercoiled pBR322 DNA or $^{32}$P-labeled DNA fragment, an LAHPO or LA in a total volume of 100 $\mu$l. After incubation at 37°C for 1 h, the mixture was subjected to ethanol precipitation to remove LAHPO and the DNA recovered was applied in agarose gel electrophoresis or sequence analysis.

### 2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was performed overnight on 0.8% agarose gel (Sigma type II) in the horizontal apparatus at 50 V (2.5 V/cm).

### 2.6. Nucleotide sequencing

The nucleotide sequence was determined as in [22]. The 5′-termini of DNA fragments were dephosphorylated with alkaline phosphatase, labeled with T4 polynucleotide kinase and 3′-protruding ends were labeled with Klenow polymerase. For sequence analysis, ethanol-precipitated $^{32}$P-labeled DNA treated with LAHPO or LA was degraded by heating at 90°C for 30 min in freshly prepared 1 M piperidine, lyophilized, dissolved in sequence dye, and applied to a sequence gel. Electrophoresis was performed on an 8% polyacrylamide gel containing 7 M urea at 1500 V for 3 h. Autoradiography was carried out at −70°C with the aid of an intensifying screen.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of LAHPO, LA and LAHO on pBR322 plasmid DNA

DNA cleavage of LAHPO was followed by monitoring the conversion of supercoiled pBR322 plasmid DNA (form I) to open circular and linear DNA (forms II and III, respectively). The introduction of one single-strand break converts form I to form II. As shown in fig.2a, supercoiled pBR322 plasmid DNA changed completely to a mixture of open circular and linear DNA after 3 h incubation with LAHPO at 37°C. Thus, LAHPO caused DNA strand breakage. On the other hand, neither LA nor LAHO caused DNA decomposition. These results indicate that the hydroperoxy group of LAHPO contributes to dsDNA decomposition. The pBR322 DNA treated with LAHPO or LA was digested with restriction enzyme (fig.2b). The DNA treated with LAHPO was cleaved by *Eco*RI (-GAATTC-) and the linear DNA fragment was found to be partially decomposed. Similar results were obtained from *Bam*HI (-GGATCC-) and *Hind*III (-AAGCTT-) digestions. Effects of LA and LAHO treatments were not observed on the DNA digestion pattern by restriction enzymes. From the above results, it seems that LAHPO interacted with DNA strand, resulting in cleavage of supercoiled DNA. However, it was not clear why LAHPO gave the adduct with DNA.

### 3.2. Fragment of dsDNA by LAHPO and piperidine treatment

The cleavage specificity on double-helical DNA by LAHPO was tested by using a DNA fragment, 473 nucleotides in length, prepared by the usual methods from plasmid pSS634 and labeled at the 3′-end with $^{32}$P. The cleavage site of nucleotide by LAHPO is indicated in fig.3,4. As shown in

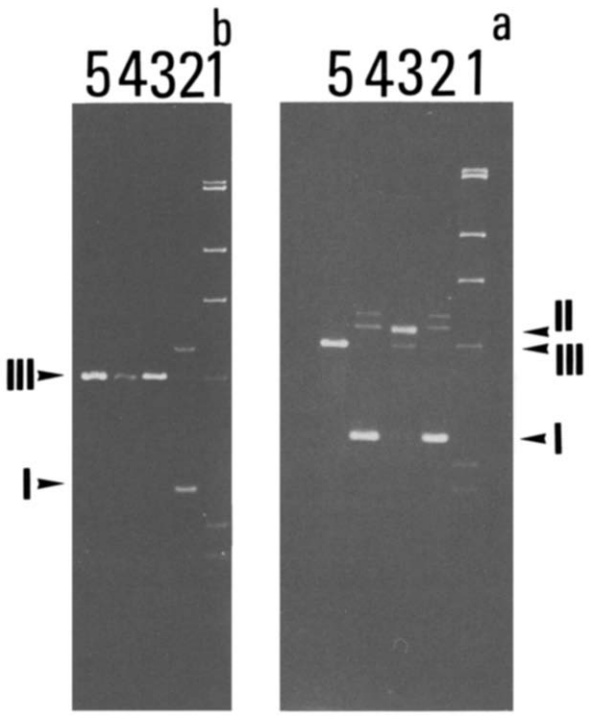


Fig.2. (a) Cleavage of supercoiled pBR322 plasmid DNA in the presence of LAHPO and (b) *Eco*RI digestion of pBR322 treated with LAHPO. (a) pBR322 (2.5 $\mu$g), LA or LAHPO (6 $\mu$mol) and the buffer were allowed to react at 37°C for 3 h. The reaction product was analyzed with agarose gel electrophoresis and photographed after ethidium bromide staining. Lanes: (1) marker $\lambda$ phage DNA-*Hind*III digestion; (2) control pBR322 DNA; (3) LAHPO/pBR322; (4) LA/pBR322; (5) pBR322-*Eco*RI digestion. (b) After removal of LA or LAHPO with ethanol, DNA was digested with *Eco*RI (4 units) in 50 $\mu$l of 50 mM Tris–HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$ at 37°C for 30 min. Lanes: (1) marker $\lambda$ phage DNA-*Hind*III digestion; (2) control pBR322 DNA; (3) pBR322-*Eco*RI digestion; (4) LAHPO/pBR322-*Eco*RI digestion; (5) LA/pBR322-*Eco*Ri digestion.

fig.3, using $^{32}$P-labeled fragment, incubation with LAHPO and LA alone (lanes 5 and 3, respectively) gave no clear bands. LA and piperidine treatment gave faint ladder bands (lane 4), but piperidine treatment alone of DNA fragment gave the same ladder bands (lane 1).

Nevertheless, the samples treated with LAHPO and piperidine gave specific band patterns in lanes 6–8. These bands were at the guanine position and the nearest neighbouring bases of guanine, which were compared with those of the corresponding

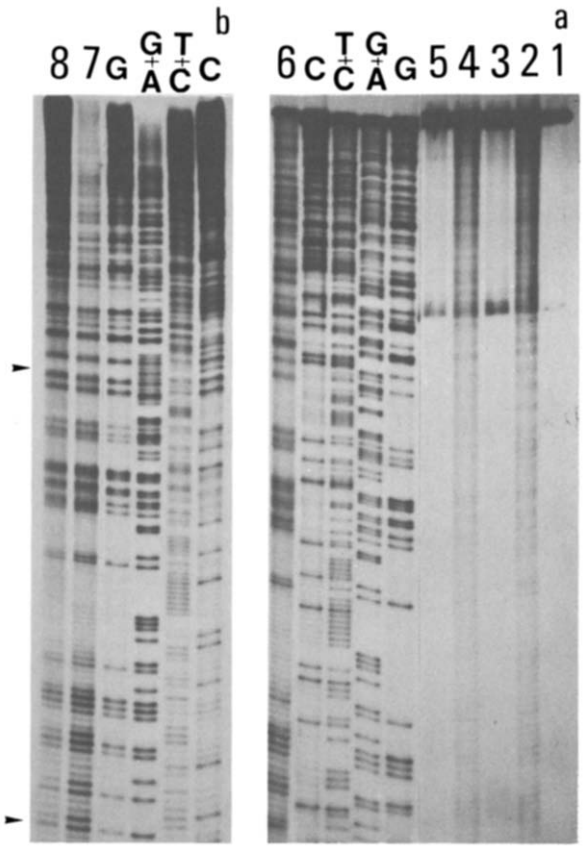


Fig.3. (a) Site-specific cleavage of dsDNA by LAHPO and (b) effect of LAHPO concentration on DNA decomposition. $^{32}$P-labeled DNA fragments were prepared from pSS634(a) and pSS604(b), respectively (a) Lanes: (1) control; (2) control + piperidine; (3) LA (6 $\mu$mol); (4) LA (6 $\mu$mol) + piperidine; (5) LAHPO (6 $\mu$mol); (6) LAHPO (6 $\mu$mol) + piperidine. (b) Lanes: (7) LAHPO (1 $\mu$mol) + piperidine; (8) LAHPO (16 $\mu$mol) + piperidine treatment. Arrows indicate sequence in fig.4.

marker sequence. However, the specificity of the nearest neighbouring base was not clear in fig.4. At lower concentration of LAHPO (lane 8), the

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    5'.....CAGACATAGCCAAACCCATAGACTAAGTGTTTAAAAAAGAACATGAAAATT
(32P)3'.....GTCTGTATCGGTTTGGGTATCTGATTCACAAATTTTTTCTTGTACTTTTAA

    GACACTACCAACCCAGAATATGACTCTGCATTTAAAAATATCCGTACCG.....3'
    CTGTGATGGTTGGGTCTTATACTGAGACGTAAATTTTTATAGGCATGGC.....5'
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Fig.4. Nucleotide sequence of fragment and cleavage site caused by LAHPO treatment. The sequence and cleavage site of the double-strand fragment taken from the autoradiography in fig.3. (*) Cleavage site on one strand of DNA restriction fragment by LAHPO.

magnitude of the relative intensity of nearest neighbouring bases of the bands on the autoradiogram decreased considerably. These results suggest that strand breakage of DNA is LAHPO-concentration dependent and that the solubility of LAHPO in the reaction mixture appears to be an important factor for DNA decomposition. Using 5'-end-labeled DNA fragment, similar results to 3'-end-labeled DNA fragments which produced the guanine specific band patterns were obtained.

The mechanism by which LAHPO brought about cleavage of dsDNA may consist of two steps. First, LAHPO interacted with guanine nucleotide of dsDNA specifically and the decomposition was further stimulated by alkaline treatment. This specificity is perhaps caused by reactivity of the hydroperoxy group of LAHPO with the purine ring of guanine, but the exact mechanism of DNA degradation by LAHPO is not clear and DNA adduct with LAHPO has also not been detected. Although the mechanism of cleavage of DNA by bleomycin [15], neocarzinostatin [16], mitomycin C [23], aflatoxin B1 [24] and phenanthroline [25] has been reported, the cleavage mechanism by LAHPO has so far been adopted. The DNA cleavage by LAHPO suggests that lipid hydroperoxides destroy the DNA molecule.

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