

IMPROVED METHOD FOR EXTRACTION OF HYDROPEROXIDE LYASE FROM *CHORELLA*

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SUMMARY

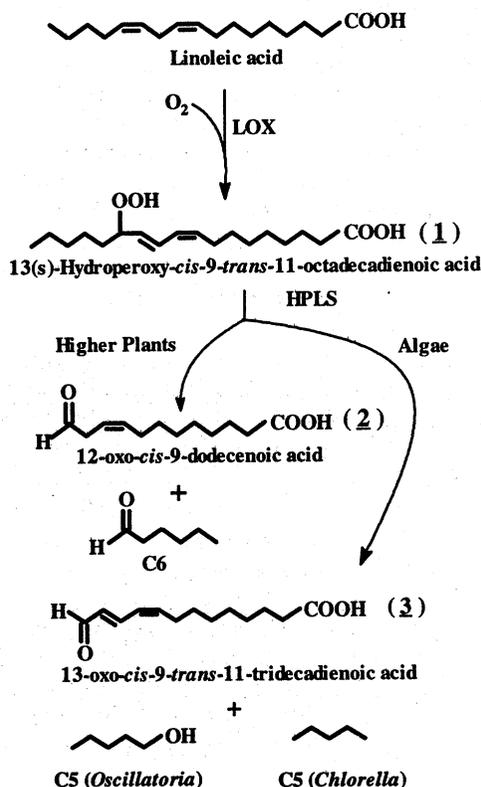
Hydroperoxide lyase converts fatty acid hydroperoxides to oxo-fatty acids which, after further oxidation, are suitable for the synthesis of higher polyamides. An improved method utilizing an acetone powder step for isolation of this enzyme from the unicellular alga *Chlorella* was developed. Using this procedure a five fold increase in hydroperoxide lyase activity from *Chlorella pyrenoidosa* was obtained compared to previously used extraction methods. Other *Chlorella* species were assayed, and it was found that *C. fusca* also contained significant hydroperoxide lyase activity.

INTRODUCTION

The hydroperoxide, 13-hydroxyperoxy-*cis*-9-*trans*-11-octadecadienoic acid, **1** (Scheme 1), obtained from the lipoxygenase (LOX, EC 1.13.11.12) catalyzed reaction of oxygen with linoleic acid (LA), can be cleaved enzymatically to an oxo-carboxylic acid by hydroperoxide lyase (HPLS). Depending upon the source of HPLS, the enzymatic cleavage of **1** produces either a C12 or C13 oxo-carboxylic acid, **2** and **3** respectively (Scheme 1), and a C6 or C5 fragment. Membrane-bound HPLS from higher plants produces the C12 oxo-carboxylic acid **2** (Gardner, 1991), whereas the water soluble HPLS from the unicellular algae *Chlorella pyrenoidosa* and *Oscillatoria* sp. gives the C13 acid **3** (Gardner, 1991; Vick and Zimmerman, 1989; Andrianarison *et al.*, 1989).

Oxidation of free LA or its glycerol esters to the corresponding hydroperoxide derivatives by LOX is a very efficient process (Piazza, 1992; Piazza and Nuñez, 1995). Moreover, the stability of LOX is improved by immobilization, thus enhancing its potential for industrial use (Piazza *et al.*, 1992; Parra-Diaz *et al.*, 1993). The sequential action of LOX and algal HPLS on LA will produce the C13 oxo-acid **3** (Scheme 1), a precursor to a C13 dicarboxylic acid. The latter dicarboxylic acid can be used to produce a polyamide similar to nylon 13,13, a polymer that has excellent dimensional stability, low affinity for moisture, high

Scheme 1



dielectric constant and good melt properties (Van Dyme and Blase, 1990).

Moreover, the double bonds in polyamides derived from **3** would allow for further cross linking or functionalization of the polymer. The enzymatic conversion of **1** to **3** would be an environmentally safe, potentially cost-effective technology, compared to the production of nylon 13,13 from a C13 dicarboxylic acid derived from the ozonolysis of erucic acid, a process which has been indicated to be too expensive to compete with other industrially produced nylons (Van Dyme and Blase, 1990).

Because HPLS from algae is water soluble rather than membrane-bound as in higher plants, algae were considered attractive sources for obtaining HPLS. However, algae cell walls are usually difficult to lyse. The enzyme extraction procedures reported in the literature for the isolation of HPLS, for the most part, use mechanical methods, and no indication of the extraction efficiency of HPLS has been given. The specific aim of this work was to develop an effective procedure for obtaining HPLS from algae and to determine which strains of *Chlorella* contain significant amounts of the enzyme.

MATERIALS AND METHODS

Materials

Soybean lipoxidase Type 1-B (LOX), linoleic acid (LA), yeast alcohol dehydrogenase (ADH), NADH and DEAE Sepharose CL-6B were purchased from Sigma (St. Louis, MO). Water was purified to a resistance of 18 M Ω -cm using a Barnstead (Dubuque, IA) NANOpure system. All other reagents were of the highest purity available.

Preparation of 13-Hydroperoxy-*cis*-9-*trans*-11-octadecadienoic Acid, **1**

1 was produced by adding four 200 μ L aliquots of a 10 mg/mL solution of LOX over 2 h to a solution of 40 mg LA in 50 mL of borate buffer (pH 9.0) at 0°C, with controlled constant oxygen bubbling at 5 mL/minute. At the end of the reaction, the mixture was acidified to pH 3.0 with HCl (1.0 M), and **1** was extracted with ethyl ether (2 x 5 mL). The combined ether layers were dried with anhydrous sodium sulfate, filtered and the ether evaporated under a stream of nitrogen. The residue was redissolved in ethanol. The extracted products were analyzed by TLC (Parra-Diaz *et al.*, 1992) and HPLC with a Waters MCL1 instrument equipped with UV detector and a Varex Mark III

evaporative light-scattering detector, and a CHROMSPHER SI (200 x 30 mm) column (Chrompack, Raritan, NJ) run isocratically with hexane:isopropanol (98:2 v/v). Results indicated that the conversion to the hydroperoxide exceeded 90%.

Algae Growth and Hydroperoxide Lyase Isolation

Chlorella pyrenoidosa (strain 211/8b) was obtained from the American Type Culture Collection (No 11469). *C. fusca* (No 251), *C. vulgaris* (No 395) and *C. sorokiniana* (No 1230) were obtained from the Culture Collection at UTEX (Austin, TX). Cells were grown in a 1 L flask containing 0.5 L of growing medium, as reported by Sorokin and Krauss (1958). Air containing ambient amounts of CO₂ was bubbled into the flasks (1-2 L/min), and the flasks were shaken at 125 cycles per minute. The algae received 16 h of light at 25°C, and 8 h of dark at 20°C. Cultures were harvested after 4 days of growth, in the late growth phase, and centrifuged at 2,000g to yield 2.5-3 g of wet cells which were washed with distilled water and lysed by either one of three methods: a) Mechanical homogenization: a PT10-35 Polytron (Brinkman Instruments, Inc. Westbury, NY) or an Ultra Turrax T25 (Janke & Unkel) homogenizer at full power for three periods of 2-4 minutes in potassium phosphate buffer (pH 7.0), at 0-4°C. b) French press: the cells were dispersed in 20-30 mL of potassium phosphate buffer (pH 7.0), compressed at 6.9x10⁶ Pa and collected at normal pressure at 0-4°C. c) Acetone powder method: cells were pelleted at 12,000g, and transferred to a blender containing 300 mL of acetone at -70°C. After 5 minutes of blending the acetone was filtered, the filter cake was washed with cold acetone under nitrogen and dried *in vacuo*.

In the first two procedures, cellular residue was removed by centrifugation at 12,000g, and the supernatant was passed through a 70 μ m nylon mesh filter. The enzyme was extracted from the acetone powder by stirring for 5 minutes with 10 mL of potassium phosphate buffer (pH 7.0). Residue was removed by centrifugation at 12,000g.

Each supernatant, obtained from the above procedures, was loaded onto a 12 x 2.5 cm DEAE Sepharose CL-6B column, and HPLS was obtained by isocratic elution with 50 mM potassium phosphate (pH 7.0) containing 15 mM NaCl. Fractions (3.5 mL) were collected until a total of 30-40 fractions were obtained.

HPLS Assay

HPLS activity was assayed by monitoring the loss of absorption at 234 nm. A typical assay mixture consisted of 30 M **1** in 50 mM potassium phosphate (pH 7.0) and 200-400 μ L of enzyme extract in a total volume of 1 mL. One unit (U) is defined as the loss of one nmol of substrate per minute. The concentration of **1** was determined by using an extinction coefficient of 23,000 $M^{-1}cm^{-1}$ (Gibian and Vandenberg, 1987). Specific HPLS assays at 340 nm with yeast alcohol dehydrogenase (ADH) and NADH were performed as described by Vick (1991). Protein concentration was determined by the dye-binding method of Bradford (1976).

RESULTS AND DISCUSSION

Cell Disruption Efficiency

Previous work with *C. pyrenoidosa* and *Oscillatoria sp.* used mechanical homogenization for the breakage of cells (Vick and Zimmerman, 1989; Andrianarison *et al.*, 1989). However, microscopic inspection of the homogenized cultures of *C. pyrenoidosa* showed that only a small percentage of the cells were lysed by this method. Homogenization was particularly poor for the *C. vulgaris* strain, and a more effective procedure was needed to increase recovery of HPLS. Accordingly, cells also were lysed by passing them through a French press or by preparing an "acetone powder" with cold acetone. Fig. 1 shows the protein and HPLS elution profiles from DEAE Sepharose of the buffer extracts of the three HPLS extraction methods. As seen in Fig. 1b, after French press treatment both the total protein and HPLS activity increased substantially compared to homogenization (Fig. 1a). These results were consistent with microscopic observations that showed few intact cells remained after French press treatment. However, HPLS activity was found only in those fractions that eluted after the bulk of the total protein present, possibly because protease or HPLS inhibitors also were liberated by the French press method.

Table I
Lysis method comparison for *C. Pyrenoidosa*
HPLS after DEAE purification.

	Vol. (mL)	U/mL	Yield (U)	Spec. act. (U/mg prot.)
Homogenization	24.5	1.11	27.2	216
French Press	14.0	3.60	50.3	265
Acetone Powder	35.0	4.24	149.0	1,050

The acetone powder method (Fig. 1 c) was found to be most effective for extraction of HPLS. Although the amount of extracted protein was similar to that obtained by the homogenization method, HPLS activity nearly quintupled, as can be seen from the increase of specific activity (U/ mg of protein) in Table I.

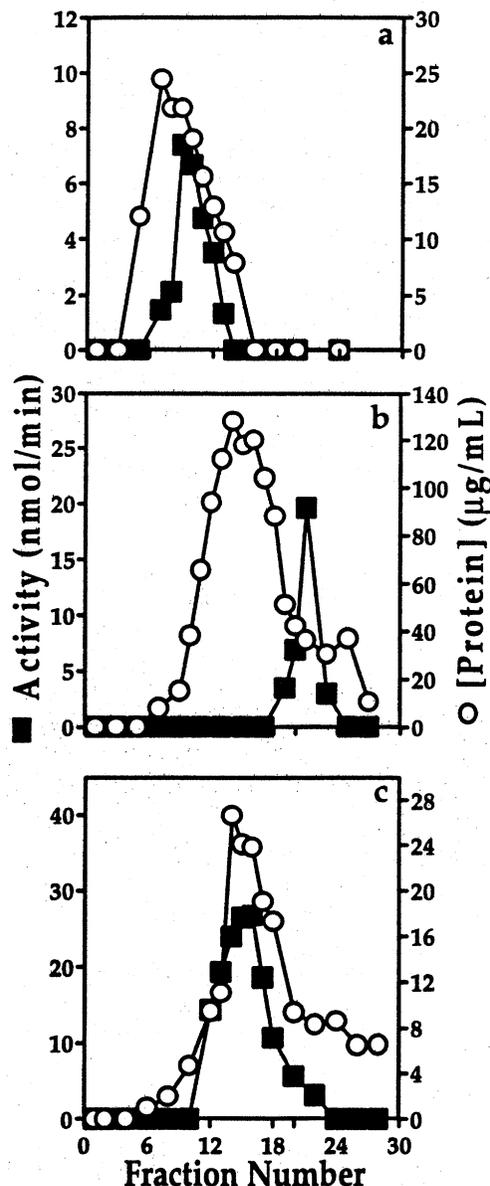


Figure 1: Comparison of HPLS activity (■) and protein concentration (○) after elution of *C. pyrenoidosa* extracts from DEAE Sepharose. The extracts were obtained by a) homogenization, b) French press, and c) acetone powder preparation.

Another benefit of the acetone powder preparation is that acetone removes the bulk of the chlorophyll, and the cellular extract could be assayed directly for HPLS activity without subjecting it to ion exchange chromatography, as was required in the other extraction procedures. Acetone powder could be stored for several days at -10°C without significant loss in HPLS activity.

HPLS in *Chlorella* Strains

In earlier studies, it was found that *Chlorella pyrenoidosa* and *Oscillatoria* sp. contained a heat labile, water soluble HPLS (Vick and Zimmerman, 1989; Andrianarison, *et al.*, 1989). No other strains of algae have been reported to contain HPLS. We have grown *C. vulgaris*, *C. sorokiniana* and *C. fusca* and assayed them for the presence of HPLS using *C. pyrenoidosa* as a reference. The *C. vulgaris* and *C. sorokiniana* strains did not contain detectable levels of HPLS. In contrast, *C. fusca* yielded 160 U HPLS, comparable to that of *C. pyrenoidosa* (149 U, see Table I) after cycling the cultures four times. After partial purification, HPLS isolated from *C. fusca* retained its activity up to six days at room temperature.

HPLS Activity

Enzyme assays were performed by following the decrease in absorption at 234 nm. This method is not specific to HPLS, because it does not differentiate between HPLS and hydroperoxide dehydrase, which will also cause a decrease in absorbance at 234 nm. A specific spectrophotometric assay for HPLS based upon the ability of yeast alcohol dehydrogenase (ADH) to reduce aldehydes to alcohols in the presence of NADH has been reported by Vick (1991). Initial rates, measured at 234 nm, were comparable with those determined with ADH and NADH at 340 nm, indicating that hydroperoxide dehydrase was not contaminating our HPLS preparations. A similar result was reported with *C. pyrenoidosa* (Vick and Zimmerman, 1989).

Conclusion:

The acetone powder method for disrupting algae cells is an efficient method for obtaining water soluble HPLS extracts. With this method, *C. pyrenoidosa*, *C. fusca*, *C. vulgaris* and *C. sorokiniana* were screened for the presence of HPLS. After DEAE Sepharose purification, only the extracts derived from the *C. fusca* and *C. pyrenoidosa* strains were found to have levels of HPLS that are adequate for subsequent production

of the desired 13-oxo-9,11-tridecadienoic acid synthase. Current research in the application of HPLS to this goal is under study.

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