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PURIFICATION AND PROPERTIES OF LIPOXYGENASE IN *MARCHANTIA POLYMORPHA* CULTURED CELLS

KENJI MATSUI,* HIROKO NARAHARA, TADAHIKO KAJIWARA and AKIKAZU HATANAKA

Department of Agricultural Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan

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Key Word Index—*Marchantia polymorpha*; Marchantiaceae; liverwort; purification; lipoxygenase.

Abstract—Lipoxygenase activity has been detected in cultured cells of a bryophyte, *Marchantia polymorpha* (liverwort). The activity was enhanced in the stationary phase. Most of the activity occurred in the cytosolic fraction. The lipoxygenase was purified to homogeneity by ammonium sulphate fractionation and hydrophobic, ion-exchange, and gel filtration chromatography. From the SDS-polyacrylamide gel electrophoresis, the M_r of the lipoxygenase was estimated as 109 600. The pH optimum was *ca* 9.0 and the p*I* value was 4.8. This enzyme formed mainly 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid from linoleic acid. It showed twice the activity for γ-linolenic acid than for linoleic acid.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) is a dioxygenase which catalyses the conversion of polyunsaturated fatty acids containing a 1,4-(*Z*,*Z*)-pentadiene system into the conjugated hydroperoxy fatty acids. This enzyme is widely distributed in a great variety of higher plants [1], nevertheless, its physiological role in higher plants has not been fully elucidated.

In the course of our comprehensive survey for an enzyme source suitable to elucidate the role of lipoxygenase in plant cells, we first detected lipoxygenase activity in cultured cells of *Marchantia polymorpha*. Not only higher plants but also lower plants such as blue-green and green algae have lipoxygenase activity [2, 3]. But it is not known if the properties of the enzymes in lower plants are the same as those in the higher plants. We therefore purified and characterized the lipoxygenase from *M. polymorpha*. To the best of our knowledge, there has been no published report on lipoxygenase activity in bryophytes.

RESULTS AND DISCUSSION

Subcellular localization

Lipoxygenase activity was depleted by boiling the crude extract for 10 min. The cells cultured for 13 days were harvested and homogenized with a Polytron mixer with three volumes of 67 mM K-Pi, pH 7.2 containing 0.4 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1% (w/v) bovine serum albumin, 1 mM EDTA and 10 mM ascorbic acid. After filtration through cheesecloth, and centrifugation of the filtrate (80 000 *g*, 80 min) over 90% of the total lipoxygenase activity was found in the supernatant. The chloroplastic fraction (1200 *g* ppt.) had *ca* 8% of the activity. The activity in the chloroplastic fraction was not reduced by repeated washes with hypotonic medium, but brief sonication (10 sec × 4) solubilized all the activity. This suggested that a part of the lipoxygenase activity is loosely bound to the chloroplastic membrane. The occurrence of lipoxygenase activity in the chloroplastic membrane was reported for tea [4] and wheat leaves [5]. In this report, the cytosolic lipoxygenase in *M. polymorpha* cells has been purified and characterized.

Time course

In order to reveal the time course of growth and lipoxygenase activity of the cells, 6 g fr. wt of the cells were grown in fresh culture medium (100 ml) and harvested every 2 days. After about four days of a lag phase cells grew logarithmically for a further six days; thereafter, the fr. wt of the cells decreased (Fig. 1A). Although high lipoxygenase activity was obtained at the start of the culture, the activity rapidly decreased and well-growing cells in logarithmic phase showed the lowest activity (Fig. 1B). Lipoxygenase activity increased to the original level when growth of the cells had almost ceased. A similar time course of the activity was obtained when the activity was expressed on the basis of protein content. This result suggests an essential role of lipoxygenase activity in a later stationary phase, but not in the other phases, of the cell growth.

Purification

A single peak of activity was obtained with hydrophobic chromatography of the ammonium sulphate fraction and then ion exchange chromatography on a QA 824 column (Table 1). Gel filtration chromatography on a TSKgel G3000SW column efficiently purified the lipoxygenase to homogeneity (Fig. 2). With this purification step, the lipoxygenase was purified 531-fold with a yield

*Author to whom correspondence should be addressed.

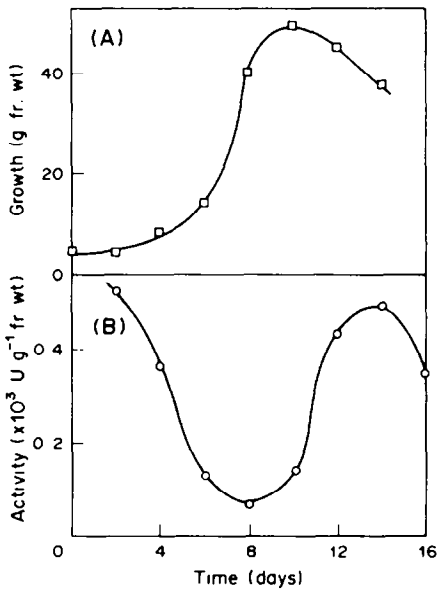

Fig. 1. Time course of growth (A) and lipoxygenase activity (B) of *M. polymorpha* cultured cells. After transplanting the cells to fresh culture medium, the cells were harvested every two days and fr. wt per 100 ml culture medium (□) and lipoxygenase activity (○) were determined.

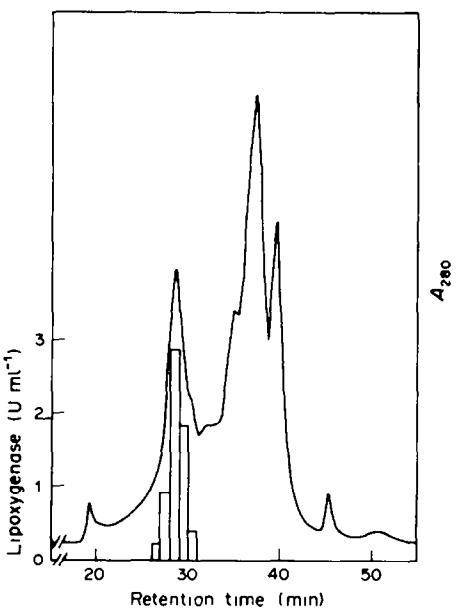

Fig. 2. Purification of *M. polymorpha* lipoxygenase with gel filtration chromatography on a TSKgel G3000SW column. The active fractions of ion exchange chromatography were collected and concentrated, then applied to the column. The lipoxygenase was eluted with 0.1 M Na-Pi, pH 7 containing 0.1 M Na_2SO_4 at a flow rate of 0.5 ml min^{-1} with detection at 280 nm. The bar represents lipoxygenase activity.

of 5.9% (Table 1). Specific activity of the purified lipoxygenase was estimated as 15.3 U mg^{-1} protein with linoleic acid as a substrate. This value was almost equivalent to that reported for avocado lipoxygenase [6], but almost one order of magnitude smaller than those of potato tuber [7], rice embryo [8] or soybean seed lipoxygenases [9]. The lipoxygenase showed only one distinct protein band on SDS-polyacrylamide gel electrophoresis and a M_r of 109 600 was calculated from the mobility of the lipoxygenase in relation to those of marker proteins.

General properties

Purified lipoxygenase had an optimum pH at *ca* 9.0 and half-maximum activity was obtained at pH 6.5 and 10.0. This optimum pH was anomalous as most of the pH optima of plant lipoxygenases have been reported to be 5.5–7.0. Soybean lipoxygenase-1 (pH optimum of this isoenzyme is 9.0–10.0) is a well-known exception [9]. Chromatofocusing with a PBE 94 column revealed the p*I* value of *M. polymorpha* lipoxygenase as 4.8. This value is relatively small when compared with those of the isoenzymes of soybean lipoxygenase (5.68, 6.25 and 6.15 for isoenzyme-1, -2 and -3, respectively [1]) or avocado lipoxygenase (5.9) [6]. The K_m value of *M. polymorpha* lipoxygenase obtained with linoleic acid as a substrate under the standard conditions was estimated as 18.4 μM. This value was almost equivalent to that reported for soybean lipoxygenase-1 [9]. The presence of 1 mM nordihydroguaiaretic acid or 1 mM mercuric chloride inhibited 100 and 80% of the activity of *M. polymorpha* lipoxygenase, respectively. Both phenidone (16.7 μM) and α-tocopherol (16.7 μM) inhibited 55% of the activity. Neither 1 mM KCN, NaN_3 nor EDTA inhibited. These results are almost the same as those obtained with lipoxygenases of higher plants [1].

Table 1. Purification of lipoxygenase from *M. polymorpha* cells

	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Enrichment (fold)
Crude extract	1470	42.2	2.88×10^{-2}	100.0	1
80 000 *g* supernatant	705.0	57.9	8.21×10^{-2}	137.0	3
$(NH_4)_2SO_4$ fraction	212.0	52.1	0.246	123.0	9
Butyl Toyopearl	18.0	23.7	1.32	56.2	46
QA-824	1.24	6.6	5.35	15.6	186
G3000SW	0.164	2.5	15.3	5.9	531

Table 2. Substrate specificity of *M. polymorpha* lipoxygenase

Fatty acids	Relative activity (%)
18:2 (*n*-6)	100.0
18:3 (*n*-6)	200.0
18:3 (*n*-3)	33.7
20:4 (*n*-6)	58.3
20:4 (*n*-3)	75.0
20:5 (*n*-3)	31.6
22:6 (*n*-3)	37.5
Linoleyl alcohol	10.9
Phosphatidyl choline	5.4

Substrate and product specificity

All the fatty acids used were dissolved in 0.2% Tween 20 soln and sonicated for 30 sec just before use. *Marchantia polymorpha* lipoxygenase was the most active with γ-linolenic acid [18:3 (*n*-6)] although the positional isomer, α-linolenic acid [18:3 (*n*-3)] was a poor substrate (Table 2). Such a high reactivity to γ-linolenic acid is not known for plant lipoxygenases. For example, with γ-linolenic acid the activities of soybean lipoxygenase-1, Bengal gram lipoxygenase-1 and -2 were 39.3, 49 and 17% of those obtained with linoleic acid, respectively [10, 11]. Polyenoic fatty acids having a chain length of C_{20} and C_{22} were also oxygenated by the enzyme, but to lesser extents.

The product specificity of the purified lipoxygenase was analysed with straight- and chiral-phase HPLC. By using linoleic acid as a substrate, the lipoxygenase formed 13-hydroperoxy-(9*Z*,11*E*)-, 13-hydroperoxy-(9*E*,11*E*)-, 9-hydroperoxy- (10*E*,12*Z*)- and 9-hydroperoxy-(10*E*,12*E*)-octadecadienoic acid in the ratio of 86.5, 4.5, 8.5 and 0.4%, respectively. Of these, the optical isomer ratio (*R*/*S*) of the 13-(*Z*,*E*)-isomer was estimated as 2/98. These results are almost coincident with those of soybean lipoxygenase-1 [9].

EXPERIMENTAL

Materials. Marchantia polymorpha cells [12] were kindly provided by Dr K. Ohyama (Kyoto University). The cells were grown under continuous fluorescent light at 25° in 100 ml of 1-M51C medium [12] in a 300 ml flask with shaking at 120 rpm. The stock cultures were subcultured every 14 days. Fr. wt of the cells was determined after filtration through filter paper under red. pres. for 5 min.

Linoleic acid (99% pure, from Sigma) was purified with a silica gel column before use. Linoleyl alcohol was prepared from linoleic acid by reduction with $LiAlH_4$. The other fatty acids (99% pure) were kindly provided by Nippon Oil and Fats Co. L-α-phosphatidylcholine (Sigma, type IV-S) was further purified with repetitive acetone pptn to remove free fatty acids.

Purification. A cell suspension (500 ml) was harvested during late stationary phase (197 g fr. wt) and homogenized with 1/4 McIlvaine's buffer, pH 8 containing 0.5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 4% (w/v) Polyclar AT. The homogenate was centrifuged at 27 000 rpm (Hitachi, RP-30-2 rotor) for 80 min and to the resultant supernatant solid $(NH_4)_2SO_4$ was added and the fr. obtained at 40–70% satn collected. The $(NH_4)_2SO_4$ fr. was redissolved in a minimum vol. of 50 mM Tris–HCl, pH 8.5 containing 25% satn of $(NH_4)_2SO_4$ and applied to a Butyl-Toyopearl 650 M column (9 × 1.9 cm, Tosoh, Tokyo) equilibrated with the same buffer. Lipoxygenase was eluted with the same buffer in which the concn of $(NH_4)_2SO_4$ was decreased to zero. Active frs were pooled and dialysed against 100 vol. of 50 mM Tris–HCl, pH 8.5 for 4.5 hr with three changes. The following steps were carried out with HPLC (Hitachi L-6200). The dialysate was applied to a QA-824 column (75 × 8 mm, Showa Denko, Tokyo) equilibrated with the dialysing buffer and the lipoxygenase was eluted with 0–0.5 M KCl gradient constructed with the same buffer. The active frs were collected and concn with Centricon-30 (Amicon) and applied to a TSKgel G3000SW column (600 × 7.5 mm, Tosoh, Tokyo) equilibrated with 0.1 M Na–Pi, pH 7.0 containing 0.1 M Na_2SO_4 and eluted with the same buffer.

Lipoxygenase assay. The standard assay mixt. (3 ml) consisted of 50 mM borate buffer, pH 9.0 and 25 μl of substrate soln (10 mM linoleic acid dissolved in 0.2% Tween 20) and appropriate vol. of the enzyme soln. The reaction was initiated by the addition of the substrate soln and formation of linoleic acid hydroperoxide was followed with the *A* at 234 nm (ε 2.5 × 10^4 M^{-1} cm^{-1}) at 25°. One unit of the enzyme activity was defined as the enzyme forming 1 μmol of the product per min.

Analysis of reaction products. Linoleic acid (5 mg) was incubated with 0.5 U of the purified lipoxygenase in 50 mM borate buffer, pH 9 at 25° for 15 min. After the formed hydroperoxide was extracted with hexane–Et_2O (1/1), reduced with $NaBH_4$ and esterified with CH_2N_2, the positional and the geometrical isomers were analysed in a form of methyl hydroxy-linoleate with HPLC (Shimadzu LC-5A) equipped with a Zorbax-SIL column (250 × 4.6 mm, Shimadzu, Kyoto; *n*-hexane–EtOH (199/1), flow rate 2 ml min^{-1}, detection at 234 nm). The methyl hydroxy-ester derivative of the 13-(*Z*,*E*)-isomer was collected and the optical isomer ratio analysed with HPLC equipped with a Chiralcel OB column (250 × 4.6 mm, Daicel, Tokyo; *n*-hexane–EtOH (99/1), flow rate 1 ml min^{-1}).

Other methods. Protein content was determined with BCA protein assay reagents (Pierce) with bovine serum albumin as standard [13]. Isoelectric point was determined by chromatofocusing on PBE 94 (Pharmacia) column as described in the instruction manual.

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