## Separation of 13- and 9-Hydroperoxide Lyase Activities in Cotyledons of Cucumber Seedlings

Kenji Matsui, Yasushi Shibata, Tadahiko Kajiwara, and Akikazu Hatanaka

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

Z. Naturforsch. **44c**, 883–885 (1989); received April 25, 1989

Cucumber Cotyledons, Hydroperoxide Lyase, Fatty Acid 13-Hydroperoxide, Fatty Acid 9-Hydroperoxide, Short-Chain Aldehydes

In cucumber cotyledons, both $C_6$- and $C_9$-aldehyde were formed *via* hydroperoxide (HPO) lyase activity. Because it has not been elucidated whether these activities are attributed to one enzyme which can cleave both 13- and 9-HPO or to two or more enzymes each of which specifically cleaves 13- or 9-HPO, an attempt to separate HPO lyase activity was done. Ion exchange chromatography separated this activity into two fractions, one of which specifically cleaved 13-hydroperoxylinoleic acid and the other specifically cleaved the 9-isomer. 13-HPO-specific activity was most active at pH 8.0 and 9-HPO-specific one was at pH 6.5. SH-reagents inhibited both the lyases but to different extents.

### Introduction

The volatile aldehydes of chain length $C_6$ and $C_9$ are formed from the 13- and 9-hydroperoxide (HPO) of an unsaturated fatty acid, respectively, which is formed by the oxygenation reaction of lipoxygenase on a $C_{18}$ fatty acid containing 1*Z*,4*Z*-pentadiene system such as linoleic and linolenic acid [1]. This cleavage reaction is catalyzed by HPO lyase and this enzyme is widespread in plants. Recently the occurrence of this enzyme activity in animal cells was reported [2].

Previously, we have reported that cucumber seedlings have both the activities of 13- and 9-HPO lyase [3]. Matthew and Galliard also reported the existence of both the activities in *Phaseolus vulgaris* leaves [4]. But it has not been elucidated yet whether these activities are attributed to one enzyme which can cleave both 13- and 9-HPO or to two or more enzymes each of which specifically cleaves 13- or 9-HPO. The work presented here provides evidence for the existence of at least two HPO lyases differing in substrate specificity.

*Abbreviations:* 13-HPO, 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; 9-HPO, 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonylfluoride; PEG, polyethyleneglycol; HPLC, high performance liquid chromatography.

Reprint requests to K. Matsui.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0900–0883 $ 01.30/0

### Materials and Methods

Cucumber (*Cucumis sativus* L. *cv.* Suyo) seeds were soaked overnight in tap water and germinated under fluorescent light (7000 lx) with a 14 h-photoperiod at 25 °C. 9-HPO and 13-HPO were prepared from linoleic acid, using potato lipoxygenase [5] and soybean lipoxygenase (Sigma, type I) [6]. Both the HPOs contained less than 10% of geometrical and positional isomers. 13-HPO lyase activity was determined by the head space vapor method [7] and 9-HPO lyase activity was determined from the amount of the 2,4-dinitrophenylhydrazone derivatives of the products by HPLC analysis [3]. For quantification, calibration curves were constructed with authentic hexanal and (*E*)-2-nonenal (Wako Pure Chemicals, Osaka). For determination of 13-HPO lyase activity enzyme solution was incubated for 3 min at 25 °C with 5 μmol of 13-HPO suspended into an appropriate buffer, but for 9-HPO lyase activity it was incubated for 15 min at 25 °C with 9-HPO (5 μmol) because preliminary experiments showed that the reaction rate of 13-HPO lyase saturated faster than that of 9-HPO lyase. The incubation was stopped when the rate of aldehyde formation was still linear. Protein was determined by the method of Bradford [8] standardized with bovine serum albumin.

Cucumber cotyledons (6-day-old) were homogenized with 3 volumes of 50 mM phosphate buffer, pH 8.0 containing 4% Triton X-100 and 2 mM sodium ascorbate in a chilled mortar and filtered through four layers of cheese-cloth. The filtrate was stirred for 1 h at 0 °C and centrifuged at 20,000 × *g* for 10 min. The supernatant (20 ml) was filled up to 100 ml with 50 mM phosphate buffer, pH 7.0 and solid PEG 6000 was added to 8% (w/v). After incubation for 1 h on ice, the PEG solution was centrifuged at 20,000 × *g* for 30 min. The resultant supernatant was made to 22% (w/v) solution of PEG 6000 and stirred for 1 h on ice. The precipitate collected by centrifugation at 20,000 × *g* for 30 min was dissolved in a small portion of elution buffer (5 mM phosphate buffer, pH 7.5 containing 0.1% Triton X-100, 10% glycerol, 0.1 mM EDTA, 2 mM sodium ascorbate and

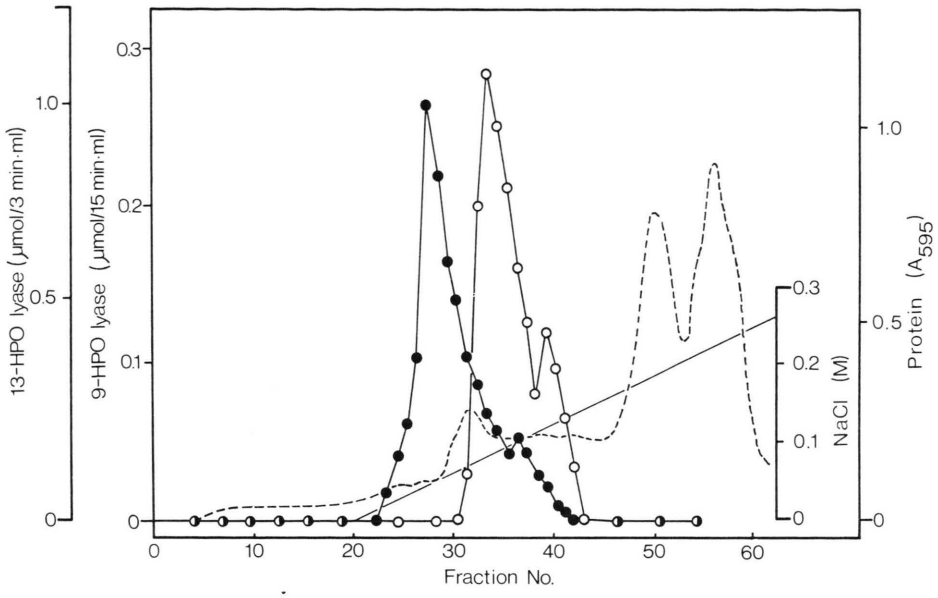


Fig. 1. Separation of 13-HPO lyase (●) and 9-HPO lyase (○) activities from cucumber cotyledons on DEAE Cellulofine A-500 (1.7×17 cm). (– – –), Protein content estimated by the method of Bradford [8].

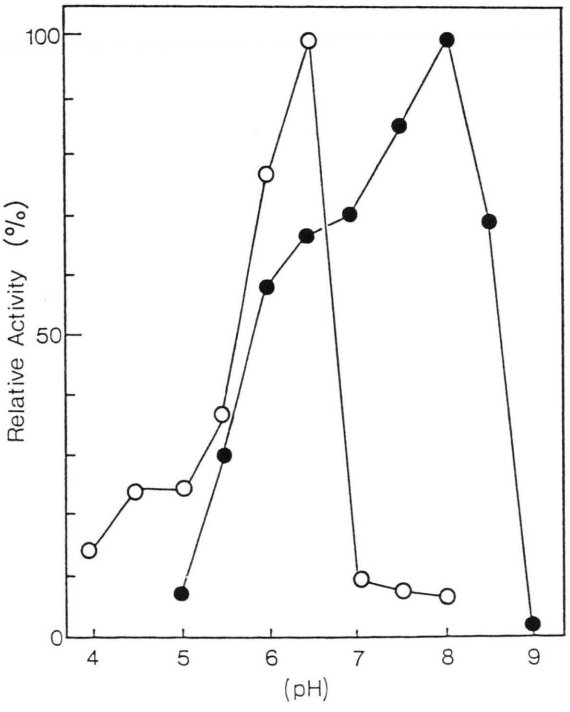


Fig. 2. pH-Dependence curve of the partially purified HPO lyases. (●), 13-HPO lyase activity; (○), 9-HPO lyase activity. McIlvaine's buffer (a mixture of 0.1 M citric acid and 0.2 M disodium phosphate, pH 4.0–8.0) and 50 mM sodium pyrophosphate buffer (pH 8.0–9.0) were used.

5 mM dithiothreitol) and put on a DEAE Cellulofine A-500 column (∅ 17×170 mm, Seikagaku Kohgyo, Tokyo) equilibrated with the elution buffer. After the column was washed with 100 ml of the elution buffer HPO lyases were eluted with 400 ml of a linear gradient of NaCl (0–0.3 M) in the elution buffer.

## Results and Discussion

Although 13- and 9-HPO lyase activities could be detected in cotyledons, hypocotyl and roots of 6-day-old cucumber seedlings, cotyledons were used as an enzyme source because this organ had the highest activity of both the lyases. Preliminary experiments revealed that 80 and 50% of 13- and 9-HPO lyase activity, respectively, were bound to a fraction precipitable with a centrifugation at 100,000×$g$ for 60 min and that 4% of Triton X-100 was necessary to extract both the activities efficiently. The extract was fractionated with PEG 6000 and applied on a DEAE Cellulofine A-500 column. Both the activities were adsorbed to this resin and could be eluted with a NaCl gradient. As shown in Fig. 1, 13-HPO lyase activity was eluted first and 9-HPO lyase activity was eluted thereafter. Although further attempts to separate these activities failed because of an instability of

Table I. Effect of reagents on 9- and 13-hydroperoxide lyase.

| Reagents | Relative activity [%] | | | |
|---|---|---|---|---|
| | 9-HPO lyase | | 13-HPO lyase | |
| | 0.1 mM | 1 mM | 0.1 mM | 1 mM |
| No added | 100 | 100 | 100 | 100 |
| EDTA | 85 | 80 | 98 | 98 |
| Monoiodoacetate | 76 | 30 | 98 | 92 |
| Mercaptoethanol | 96 | 93 | 104 | 77 |
| PCMB | 53 | 13 | 87 | 1 |
| PMSF | 80 | 40 | 95 | 85 |

9-HPO lyase, the elution profile clearly showed that at least two different enzymes were existed in cucumber cotyledons each of which was specific to 13- or 9-HPO. By this purification step 13-HPO lyase was purified about 9-fold in 61% yield and 9-HPO lyase was purified 18-fold in 82% yield.

Some properties of these partially purified lyases were also examined. As shown in Fig. 2, 13-HPO lyase had a pH optimum at 8.0 while 9-HPO lyase at pH 6.5. In both cases higher pH values rapidly decreased the activities. As shown in Table I, both the activities were much affected by PCMB which is known as an inhibitor for HPO lyase in several plants [9, 10]. Monoiodoacetate decreased 9-HPO lyase activities whereas 13-HPO lyase activity was hardly affected. A serine protease inhibitor, PMSF, also decreased 9-HPO lyase activity, whereas 13-HPO lyase activity was little affected.

These results show that in cucumber cotyledons $C_6$- and $C_9$-aldehydes were formed by different enzymes. It is well known that the substrate of HPO lyase is offered by lipoxygenase activity. We have previously reported that lipoxygenase activity which formed 13- and 9-HPO in the ratio of 85/15 from linoleic acid rapidly increased after germination and reached a maximum by the fifth day of germination. Both HPO lyases also showed almost the same developmental course of activities as that of lipoxygenase (results not shown). This coordinative change of lipoxygenase and HPO lyase activities was also reported in watermelon seedlings [10] and cotton seedlings [12].

[1] A. Hatanaka, T. Kajiwara, and J. Sekiya, Chem. Phys. Lipids **44,** 341 (1987).
[2] W. C. Glasgow, T. M. Harris, and A. R. Brash, J. Biol. Chem. **261,** 200 (1986).
[3] A. Hatanaka, T. Kajiwara, and K. Matsui, Z. Naturforsch. **43c,** 308 (1988).
[4] J. A. Matthew and T. Galliard, Phytochemistry **17,** 1043 (1978).
[5] T. Galliard and D. R. Phillips, Biochem. J. **124,** 431 (1971).
[6] J. Sekiya, S. Numa, T. Kajiwara, and A. Hatanaka, Agric. Biol. Chem. **40,** 185 (1976).
[7] A. Hatanaka, T. Kajiwara, J. Sekiya, M. Imoto, and S. Inoue, Plant Cell Physiol. **23,** 91 (1982).
[8] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[9] P. Schreier and G. Lorenz, Z. Naturforsch. **37c,** 165 (1982).
[10] B. A. Vick and D. C. Zimmerman, Plant Physiol. **57,** 780 (1976).
[11] K. Matsui, T. Kajiwara, K. Hayashi, and A. Hatanaka, Agric. Biol. Chem. **52,** 3219 (1988).
[12] B. A. Vick and D. C. Zimmerman, Plant Physiol. **67,** 92 (1981).