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FATTY ACID HYDROPEROXIDE LYASE IS A HEME PROTEIN

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SUMMARY: Fatty acid hydroperoxide lyase (HPO lyase) is an enzyme that cleaves hydroperoxides of polyunsaturated fatty acids to form short chain aldehydes and ω-oxoacids. Spectrophotometric analyses of HPO lyase highly purified from green bell pepper fruits indicate that it is a heme protein. The heme species was revealed to be heme *b* (protoheme IX) from the absorption spectrum of the pyridine hemochromogen. Although the spectrum highly resembles that of a plant cytochrome P450, allene oxide synthase from flaxseed, CO treatment of the enzyme caused no appearance of a peak at 450 nm, which is an essential diagnostic feature of a cytochrome P450. Internal amino acid sequences determined with peptide fragments obtained from the lyase showed no homology with any reported sequences.

Fatty acid hydroperoxide lyase (HPO lyase) is an enzyme that cleaves hydroperoxides of polyunsaturated fatty acids such as α-linolenic or linoleic acids to form short chain aldehydes and ω-oxoacids. This enzyme is widely distributed in plants (1,2). HPO lyase is considered to be involved in the signal transduction systems in plants because the short chain aldehydes such as (2*E*)-hexenal and (2*E*)-nonenal formed by this enzyme are elucidated to inhibit the germination of soybean seeds (3) and to induce the phytoalexin formation in cotton bolls (4). Recently, the involvement of this enzyme in a hypersensitive reaction of kidney beans against pathogens has also been reported (5).

Because HPO lyase is a component of the 'oxylipin pathway' in plants (6), which is sometimes cited in comparison with the eicosanoid pathway in mammalian cells, and because the enzyme catalyzes an unique reaction on a biologically important compound, fatty acid hydroperoxide, elucidation of the reaction mechanism has long been expected. Fatty acid hydroperoxide can be degraded either by heat, acids, or transition metals. In some cases, the degradation proceeds via homolytic scission of the hydroperoxy group, while in the other cases, via heterolytic scission. Although, no evidence to confirm the reaction mechanism of HPO lyase has been obtained. Such an approach has been hindered by the difficulty in purification of the enzyme in sufficient amounts. Recently, we succeeded to develop a system purifying the enzyme

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from green bell pepper fruits in a relatively high amount (7). With this enzyme preparation, we found that bell pepper HPO lyase is a heme protein for the first time. This finding is thought to be a great advance to elucidate the reaction mechanism. In this article the spectrophotometric properties of the lyase is reported.

MATERIALS AND METHODS

Preparation of HPO lyase: HPO lyase I was purified as previously described (7). High performance gel filtration of the HPO lyase was carried out with a liquid chromatograph system (LC-10AD, Shimadzu, Kyoto, Japan) equipped with TSK-gel G3000SW column (7.5 mm i.d. x 600 mm, Tosoh, Tokyo, Japan) using 0.1 M sodium phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4 and 0.5% (w/v) Triton X-100R (Sigma, MO, USA) as a carrier solvent at a flow rate of 0.4 ml/min. Elution was monitored by a photodiode array detector, SPD-M6A (Shimadzu).
Enzyme assay: HPO lyase activity was determined spectrophotometrically by using 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid as a substrate in 0.1 M MES-KOH, pH 5.5 by the method of Matsui et al (8). One katal of the activity was defined as the amount of enzyme that consumed 1 mol of substrate within one second.
Heme analyses: Spectrophotometrical analyses of the purified HPO lyase were carried out with a spectrophotometer, UV-160A (Shimadzu) in 0.1 M sodium phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4 and 0.5% (w/v) Triton X-100R. Reduction of the HPO lyase was done by the addition of sodium dithionite to a final concentration of 0.2% (w/v). Pyridine hemochrome was prepared from the purified enzyme (0.41 mg/ml) by adding pyridine and then NaOH to final concentrations of 20% and 0.2 M, respectively (9). Thus, the chromogen corresponded to 0.246 mg/ml of HPO lyase. SDS-polyacrylamide gel electrophoresis of HPO lyase (6.0 μg) was performed with 10.0% (w/v) polyacrylamide gel by the method of Laemmli (10). Heme chromophore was stained by the method of Thomas et al (11) by using 3,3'5,5'-tetramethylbenzidine-H_2O_2.
Amino acid sequence analysis: HPO lyase was partially digested with protease V8 (Sigma), and the resulting fragments were separated by the methods of Cleveland et al (12). The fragments were electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, MA, USA), then stained with Amide Black 10B. The major peptide bands thus obtained were cut, and the cut pieces were applied to a automated Edman degradation with a protein sequencer, PSQ-2 (Shimadzu). Sequence homology was searched with Genetyx software (Software Development, Tokyo, Japan).

RESULTS

Cochromatography of the activity with the chromophore: Fatty acid HPO lyase in green bell pepper fruits is a membrane-bound, trimeric protein of about 170 kD consisting of a subunit of 55 kD. Of the two isoforms (HPO lyase I and II) the more abundant one, HPO lyase I, was purified 2500-fold as previously described (7). The purified enzyme was examined on a high-performance gel filtration column. As shown in Fig. 1, only one symmetrical peak of the activity was detected. The enzyme activity coeluted with the peak of A393, which suggested that the enzyme have a heme chromophore. The activity peak highly correlated also with the major peak of A280. Analysis of each fraction with SDS-polyacrylamide gel electrophoresis revealed that 55-kD protein cochromatographed with both the activity and the A393 value. No other band of protein could be detected even if the fraction having relatively high A280 value was examined. Thus, the other peaks of A280 must be originated from a detergent, Triton X-100R, or its contaminants. By using the other, an hydroxylapatite column, the activity was also cochromatographed with A393. Taken together, it is indicated that the heme chromophore is a

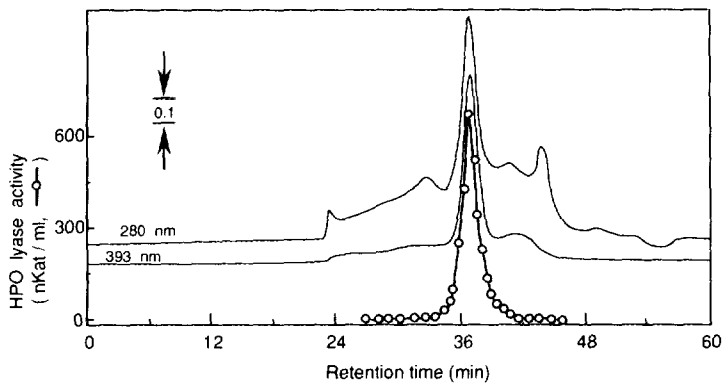

Figure 1. A representative elution profile of bell pepper HPO lyase from TSK-gel G3000SW gel filtration column. Absorptions at 280 nm originating proteins and at 393 nm originating a Soret band of a heme were continuously recorded. Fractions eluted in every 30 seconds were collected, and the activity was determined (–o–). Conditions of the chromatography and the enzyme assay were described in "Materials and Methods."

component of the enzyme. Heme staining of the enzyme after SDS-polyacrylamide gel electrophoresis (11) showed a broad positive band comigrated with bromophenol blue and a faint band comigrated with the subunit. This indicates that the chromophore does not covalently bind to the apoprotein.

Spectral properties of bell pepper HPO lyase: Our preparation of bell pepper HPO lyase exhibited an absorption spectrum (Fig. 2) very similar to that reported with β-naphthoflavone-inducible forms of liver microsomal cytochrome P450 (13) and, most prominently, to allene oxide synthase (14). Diagnostic features include the Soret band at 391 nm

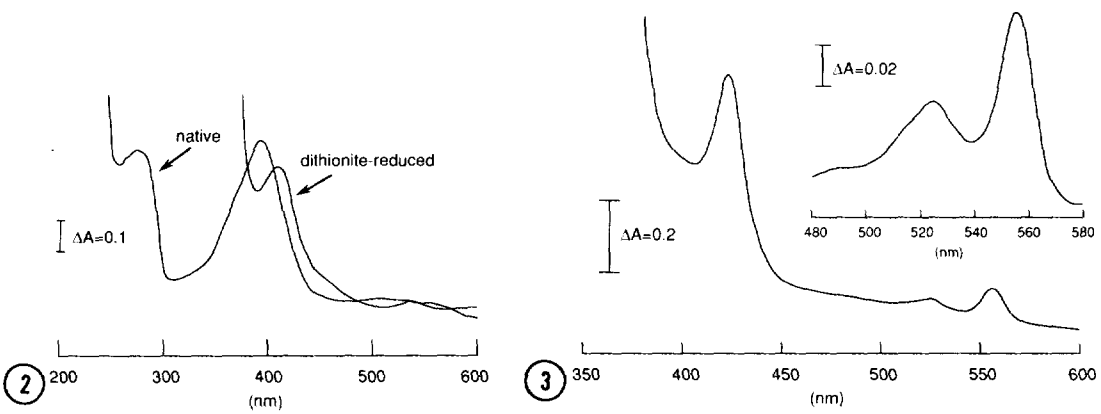

Figure 2. Absorption spectrum of HPO lyase of bell pepper. The spectrum of the native enzyme (0.41 mg / ml of protein, oxidized state) and the dithionite-reduced one in 0.1 M sodium phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4 and 0.5% (w/v) Triton X-100R.

Figure 3. Absorption spectrum of pyridine hemoferrochrome prepared from HPO lyase. The spectrum of reduced pyridine hemoferrochromogen, which is corresponding to 0.25 mg / ml of the enzyme, is shown. Inset: the difference spectrum derived from reduced minus oxidized form of the pyridine hemochrome. The sensitivity was increased about eight-fold.

and broad α band at around 500 to 540 nm. Reduction with dithionite led to the shift in the Soret band to 408 nm and broad α band to around 550 nm. Because allene oxide synthase is reported to be a cytochrome P450, we tried to obtain CO-binding spectrum of the dithionite-reduced enzyme. There was no observable change with treating the reduced enzyme with CO, which suggested that bell pepper HPO lyase is not a cytochrome P450 or, if any, it is a novel one.

Identification of the heme species: In order to ascertain the species of the heme chromophore in bell pepper HPO lyase, the pyridine hemoferrochrome was prepared. The absorption spectrum (Fig. 3) exhibited a sharp γ band at 419 nm, an α band at 556 nm and a β band at 523 nm. Difference spectrum of the hemochromogen, i.e., dithionite reduced minus non-treated, also showed distinct α band at 556 nm and β band at 523 nm. These are typical diagnostic features for heme *b* (protoheme IX) (9). From the reported extinction coefficients (A556-A539, 20.7 mM^{-1}) (15), it was indicated that the purified enzyme contained 12.4 nmol of hemes per mg of protein. Judging from the molecular mass, the HPO lyase thus appears to consist of 2.2 mol of heme *b* per mol enzyme.

Amino acid sequence analysis: The amino terminal of the enzyme was protected. Thus, the protein was partially digested with protease V8, and the resultant peptide fragments were subjected to the analysis. The internal amino acid sequences thus obtained were -SerLeuAlaArgSerProXxxValProMetGlnTyrAlaArg- and -IlePheValHisGluPheXxxTyrProTyrPheLeuValArgGlyGlyTyrGlu-. Through comparison of these sequences with those deposited in EMBL protein database with Genetyx software showed no homology with any reported sequences. As noted above the chromophore of the HPO lyase highly resembles with that of a plant cytochrome P450, allene oxide synthase. Irrespective of the similarity of the chromophore and the molecular mass (the subunit sizes of both the lyase and the synthase are 55 kD) (14), no distinct homology to allene oxide synthase (16) was observed.

DISCUSSION

In this study, several lines of evidences indicate that bell pepper HPO lyase is a heme protein. The heme species was revealed to be heme *b* (protoheme IX) from the absorption spectrum of the pyridine hemochromogen. The spectrum highly resembles with that of a plant cytochrome P450, allene oxide synthase from flaxseed (14). Allene oxide synthase is thought to be involved in the formation of jasmonic acid (6), a candidate for plant signal transducing agent (17). The enzyme acts on a fatty acid hydroperoxide formed by lipoxygenases and forms a prostaglandin-like product, 12-oxo-phytodienoic acid. With revealing the synthase as a cytochrome P450, Song and Brash proposed that the reaction of allene oxide synthase are proceeded via homolytic cleavage of the hydroperoxy group (14). Both the lyase and the synthase have the same subunit sizes of 55 kD. Irrespective of such high similarities with both the enzymes, CO treatment on the HPO lyase resulted in no change of the chromophore, thus, HPO lyase lack an essential diagnostic feature to classify the enzyme into a cytochrome P450. Nonetheless, one should wait further kinetical and structural studies before confirm whether or not the HPO lyase is a cytochrome P450 because plant P450s showing extremely low affinity to CO are recently reported (18).

From a chemical knowledge, it is suggested that the reaction of HPO lyase proceeds via a heterolytic scission mechanism where protonation of the hydroperoxy group with a Lewis acid followed by formation of carbocation and rearrangement occurs (19). Under this mechanism, hexanal and its counterpart, methyl 12-oxo-(10*E*)-dodecenoic acid are essentially sole products formed from methyl ester of linoleic acid 13-hydroperoxide. On the contrary, a homolysis caused by thermal decomposition of 13-hydroperoxide of methyl linoleate resulted in various species of products including hexanal, methyl octanoate, 2,4-decadienals and methyl azelaaldehydate (20). It is known that with iron(III) porphyrins either homolytic or heterolytic scissions of hydroperoxides occurs (21). While most peroxidases such as prostaglandin H synthase (22) cause the heterolytic scission of the peroxy O-O bond, with using a model compound it is reported that cytochrome P450s can cause both the homolytic and heterolytic scission depending on the reaction condition (23, 24). Biological homolytic cleavage of hydroperoxide of fatty acid has been reported with rabbit liver cytochrome P450 (25) and soybean hydroperoxide-dependent peroxygenase (26), both of which contain a heme *b* as a chromophore. With the former case, pentane is the major product from linoleic acid 13-hydroeperoxide. In this respect, it should be noticed that *Chlorella* HPO lyase forms pentane as one of major products (27). In order to ascertain the scission mechanism involved in HPO lyase, further study should be done. HPO lyase is 'suicide' inactivated with its own substrate, fatty acid hydroperoxide, and this inactivation can be efficiently protected with addition of radical trapping agents, such as nordihydroguaiaretic acid (8). From these knowledge, the latter, homolytic mechanism is thought to be more probable. It is noteworthy that unlike the reductant and oxygen requiring heme containing enzymes, the HPO lyase utilizes a hydroperoxide substrate and requires neither oxygen or reductant. This is also the case with allene oxide synthase (14). Anyway, precise elucidation of reaction mechanism of HPO lyase is looking forward. It should clarify a novel type of a reaction mechanism associating with heme containing enzyme.

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REFERENCES

1. Hatanaka, A., Kajiwara, T. and Sekiya, J. (1987) Chem. Phys. Lipids 44, 341-361.
2. Gardner, H. W. (1991) Biochim. Biophys. Acta 1084, 221-239.
3. Gardner, H. W., Dornbos, D. L., Jr. and Desjardins, A. E. (1990) J. Agric. Food Chem. 38,1316-1320.
4. Zeringue, H. J., Jr. (1992) Phytochemistry 31, 2305-2308.
5. Croft, K. P. C., Jüttner, F. and Slusarenko, A. J. (1993) Plant Physiol. 101, 13-24.
6. Hamberg, M. and Gardner, H. W. (1992) Biochim. Biophys. Acta 1165, 1-18.
7. Shibata, Y., Matsui, K., Kajiwara, T. and Hatanaka, A. (1995) Plant Cell Physiol. in press.
8. Matsui, K., Kajiwara, T. and Hatanaka, A. (1992) J. Agric. Food Chem. 40, 175-178.
9. Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1990) Proc. Natl. Acad. Sci. USA 87, 9863-9867.
10. Laemmli, U. K. (1970) Nature 227, 680-685.
11. Thomas, P. E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75, 168-176.

12. Cleveland, D. W., Fischer, S. G., Kirschonor, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102-1106.
13. Haugen, D. A. and Coon, M. J. (1976) J. Biol. Chem., 251, 7929-7939.
14. Song, W.-C. and Brash, A. R. (1991) Science 253, 781-784.
15. Rieske, J. S. (1967) Methods Enzymol. 10, 239-245.
16. Song, W.-C., Funk, C. D. and Brash, A. R. (1993) Proc. Natl. Acad. Sci. USA 90, 8519-8523.
17. Reinbothe, S., Mollenhauer, B. and Reinbothe, C. (1994) Plant Cell 6, 1197-1209.
18. Lau, S.-M. C., Harder, P. A. and O'Keefe, D. P. (1993) Biochemistry, 32, 1945-1950.
19. Gardner, H. W. and Plattner, R. D. (1984) Lipids, 19, 294-299.
20. Chan, H. W.-S., Prescott, F. A. A., and Swoboda, P. A. T. (1976) J. Am. Chem. Soc. 53, 572-576.
21. Labeque, R. and Marnett, L. J. (1989) J. Am. Chem. Soc. 111, 6621-6627.
22. Smith, W. L. and Marnett, L. J. (1991) Biochim. Biophys. Acta 1083, 1-17.
23. Lee, W. A. and Bruice, T. C. (1985) J. Am. Chem. Soc. 107, 513-514.
24. Traylor. T. G., Fann, W. P. and Bandyopadhyay, D. (1989) J. Am. Chem. Soc. 111, 8009-8010.
25. Vaz, A. D. N. and Coon, M. J. (1987) Proc. Natl. Acad. Sci. USA 84, 1172-1176.
26. Bleé, E. and Schber, F. (1989) Biochemistry 28, 4962-4967.
27. Vick, B. A. and Zimmerman, D. C. (1989) Plant Physiol. 90, 125-132.