Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P450 (CYP74B)

Kenji Matsui[a,*], Mizuyoshi Shibutani[a], Toshiharu Hase[b], Tadahiko Kajiwara[a]

[a]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753, Japan*
[b]*Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan*

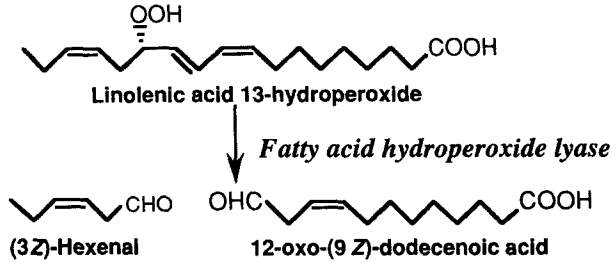
Received 4 July 1996; revised version received 8 August 1996

Abstract Fatty acid hydroperoxide lyases cleave a C-C bond adjacent to a hydroperoxide group in lipoxygenase derived lipid hydroperoxides to form short-chain aldehydes and oxo-acids. Previously, we showed that fatty acid hydroperoxide lyase from bell pepper fruits is a heme protein whose spectrophotometric properties greatly resemble a cytochrome P450. In order to ascertain the relationship of it to the P450 gene family, we have cloned cDNA encoding fatty acid hydroperoxide lyase from immature bell pepper fruits. The cDNA encodes 480 amino acids, and shares homology with P450s mostly at the C terminus. The heme binding cysteine is recognizable at position 441. The most closely related P450 is allene oxide synthase (CYP74A), with which it has 40% identity. It qualifies the lyase as a member of a new P450 subfamily, CYP74B. From this finding, the enzyme is thought to be a novel member of P450 specialized for the metabolism of lipid peroxides.

Key words: Fatty acid hydroperoxide lyase; Cytochrome P450; cDNA sequence; Fruit; *Capsicum annuum*

1. Introduction

Fatty acid hydroperoxide lyase (HPO lyase) is an enzyme that cleaves a C-C bond in the hydroperoxides (HPOs) of polyunsaturated fatty acids to generate aldehydes and ω-oxo-acids [1]. This enzyme is widely distributed in plants and is involved in the biosynthesis of volatile aldehydes and alcohols [2]. The short-chain volatile aldehydes and their reduced derivatives, alcohols, are important constituents of the characteristic flavors of fruits, vegetables and green leaves. On the other hand, their physiological roles have not been fully elucidated. Recently, Croft et al. [3] reported that (3*Z*)-hexenol and (2*E*)-hexenal were synthesized during a hypersensitive-resistance response of kidney bean. They also showed that (2*E*)-hexenal is a highly effective anti-bacterial agent. Thus, these volatile compounds have been suggested to be involved in anti-bacterial responses. Although HPO lyase has been purified from tea leaves [4] and from soybean seedlings [5] and leaves [6] and partially characterized, there remain many issues to be resolved, for example, its structure, reaction mechanism, tissue localization, changes with development, and so forth, to identify its still unknown physiological function and to reveal the mechanism to catalyze its unique reaction on biologically important lipid peroxides. To date, studies of these issues have been hampered by the relatively small amounts of the enzyme in plant tissues.



Recently, we succeeded in purifying HPO lyase from green bell pepper (*Capsicum annuum* L.) fruits in a relatively large amount [7]. Spectrophotometric analysis of the purified enzyme revealed that the enzyme is a heme protein having heme b (protoheme IX) as a prosthetic group [8]. Although the heme species and the spectrophotometric properties greatly resemble typical cytochrome P450s, bell pepper HPO lyase lacked an essential diagnostic feature to assign it as a member of P450 family, that is, the Fe(II)-CO complex having a characteristic absorption maximum near 450 nm could not be obtained even after extensive CO-bubbling. On the contrary, it is well known that some P450s, such as thromboxane synthase, prostacyclin synthase, and allene oxide synthase (AOS) act on fatty acid peroxides [9,10]. Furthermore, cleavage of hydroperoxide by P450 via homolysis of the O-O bond has also been known [11]. These evidences and the finding that bell pepper HPO lyase is a heme protein raised the question of the relationship of this enzyme to other P450s. A knowledge of the primary structure of the enzyme is an important approach to this issue.

2. Materials and methods

Immature green bell pepper fruits (*Capsicum annuum* L.) were purchased from a local market. HPO lyase was purified from the fruits as described [7]. Purified enzyme electrophoretically transferred onto Immobilon transfer membrane (Millipore Co., Bedford, MA, USA) was digested with lysil endopeptidase (*Achromobacter* protease I, Wako Pure Chemical Co., Osaka, Japan) in the presence of 7% (v/v) of acetonitrile, then the resultant peptides were separated with a reversed phase HPLC. With this procedure only relatively hydrophilic peptides could be recovered. Microsequencing was accomplished using a Shimadzu PSQ-2 gas-phase sequencer. A cDNA library constructed from poly(A)$^+$ RNA isolated from immature bell pepper fruits (3.5 g fresh weight/fruit) was immunoscreened with polyclonal antibodies directing bell pepper HPO lyase. The near full-length cDNA clone, PL22, was isolated by a subsequent screening of the cDNA library using the insert from a positive cDNA clone obtained by immunoscreening as a probe. Of 3×10^5 independent clones, 20 positives could be obtained. They shared nearly identical sequence and PL22 was the longest one. Sequence data base search was performed with the BLAST algorithm program [12]. For heterologous expression of HPO lyase, the *Nco*I-

*Corresponding author. Fax: (81) (839) 33-5820.
E-mail: matsui@agr.yamaguchi-u.ac.jp

Abbreviations: P450, cytochrome P450; HPO, hydroperoxide; AOS, allene oxide synthase; PGI, prostacyclin

PII S0014-5793(96)00924-6

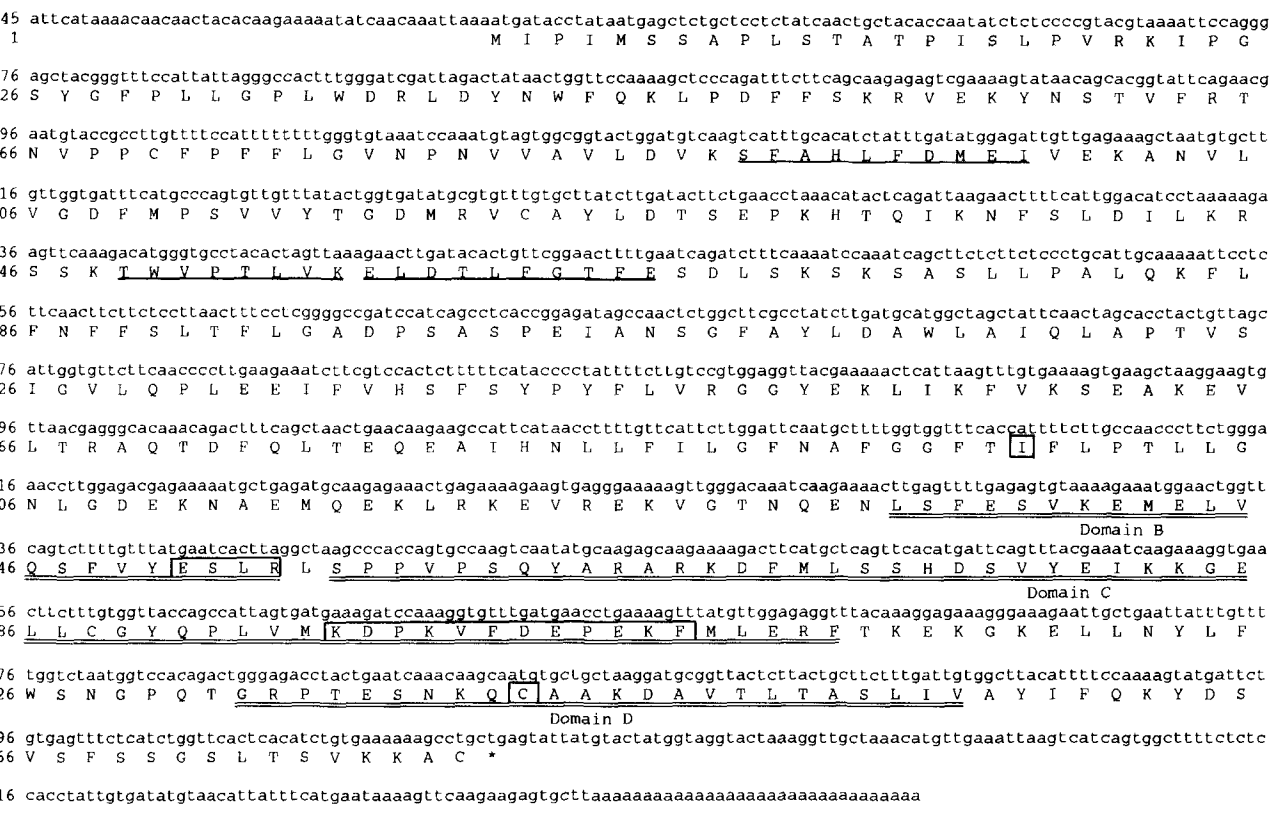


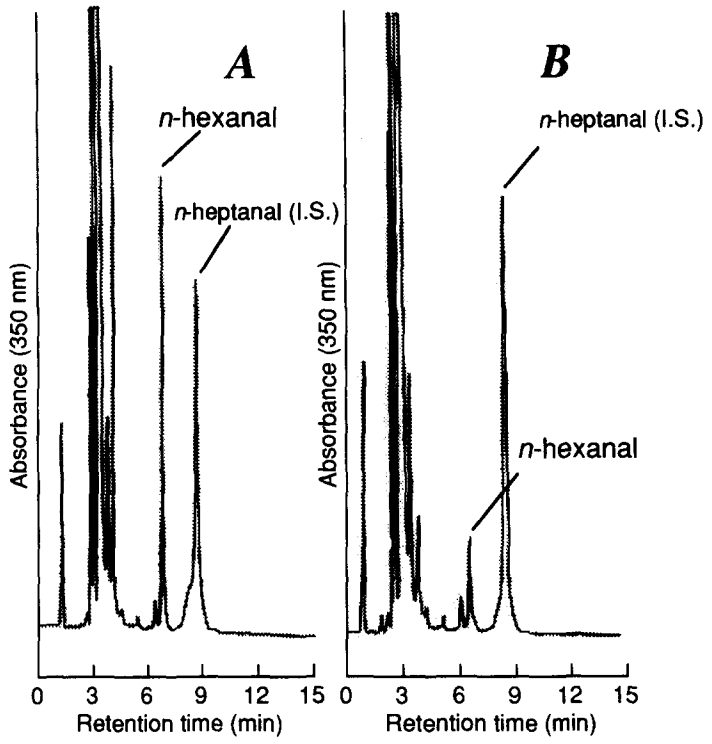
Fig. 1. The nucleotide and the deduced amino acid sequences of the bell pepper HPO lyase cDNA clones. The cDNA inserts were subcloned and sequenced on both strands by the dideoxynuleotide chain termination method. The amino acid sequences are shown below the nucleotide sequence, and the nucleotides and amino acids are numbered on the left hand side. Peptide sequences determined from the purified enzyme are underlined. The B, C, and D domains are double underlined. The heme binding C (position 441), I-298 of the I helix region, and the sequences ESLR and KDPKVFDEPEKF are boxed.

*Sac*I fragment in an *E. coli* expression vector, pTrc99A (Pharmacia), was replaced with a synthetic DNA adapter (an annealed product of 5′-CATGATACCTATAATGAGCT-3′ and 5′-CATTATAGGTAT-3′, in which the start codon is underlined), then ligated with downstream of the *Sac*I site of the coding region of PL22. The expression plasmid was transformed into *E. coli* TG1 cells. The transformant was grown in LB medium supplemented with ampicillin at 37°C until OD at 600 nm reached 1.0, then, they were further grown at 25°C for 7 h in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were harvested and disrupted with a French press in 50 mM Tris-HCl, pH 8.0 containing 1 mM EDTA, 0.1 M NaCl and 0.25 mg/ml of lysozyme. The cell lysate was centrifuged at 150 000 × *g* for 60 min to give soluble fraction and membrane fraction. HPO lyase activity was determined either by HPLC or by the ΔA234 method [7]. Western immunoblot and spectrophotometric analyses were performed according to published methods [7,8].

3. Results and discussion

3.1. Isolation of the bell pepper HPO lyase cDNA

Initial attempts to obtain the HPO lyase gene by screening a cDNA library constructed from poly(A)$^+$ RNA prepared from bell pepper fruits at an early ripening stage (generous gift from Dr. Kuntz [13]) were unsuccessful. Determination of the HPO lyase activity in the fruit at various ripening stages indicated that it was higher in younger and small fruits (data not shown). Thus, poly(A)$^+$ RNA was prepared from immature bell pepper fruits with an average weight of 3.5 g fresh weight/fruit, then a cDNA library (λgt22a) was constructed. Immunoscreening of the cDNA library allowed the isolation of six positive clones. Sequence analyses of them showed that only one clone, PF23m, had the sequences identical with the internal amino acid sequences obtained after in situ digestion of the bell pepper HPO lyase on polyvinylidene difluoride membrane by lysil endopeptidase (Fig. 1). Further screening of the cDNA library (3×10^5) with PF23m as a probe allowed the isolation of 20 positives. Their sequences were nearly identical, and therefore the longest one, PL22, was further characterized. PL22 had an insert of 1647 bp and coded 480 amino acids (Fig. 1). The protein has a molecular weight of 54 055 as calculated from the translation of the nucleotide sequence, which was almost equivalent to that of bell pepper HPO lyase [7]. Thus, it was suggested that this clone encodes nearly the full-length sequence. Sequence data base searches showed that PL22 shared homology with cytochrome P450s. Relatively high homology could be seen in the C-terminal region. The segments that are highly conserved in many P450 families, i.e. B, C, and D domains [14], could be seen in the PL22 sequence. As expected from the similarity of their catalytic properties, the bell pepper HPO lyase was most closely related to AOSs (CYP74A) [15,16] with 40% primary structural identity. These findings indicate that bell pepper HPO lyase is a member of the cytochrome P450 family. Accordingly, Nelson has officially named bell pepper HPO lyase as a member of a novel subfamily of P450, CYP74B (consequently, AOS has been renamed CYP74A, personal communication).



C

fraction	HPO lyase activity (mmol/ml culture)
Cell lysate	1.81
Soluble fraction	0.54
Membrane fraction	1.94

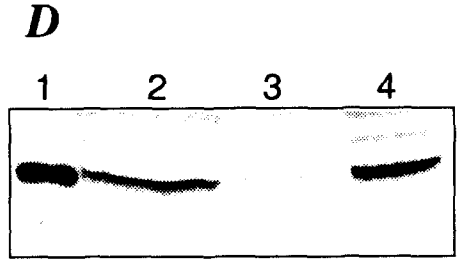


Fig. 2. Enzyme activity and immunoblot assays of bell pepper HPO lyase expressed in *E. coli*. A and B: Reversed-phase HPLC analyses of the metabolism of 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (linoleic acid 13-hydroperoxide) by lysates of transformed cells. A: HPO lyase cDNA transformed cells. B: Mock-transformed cells. The small amount of *n*-hexanal formed in mock-transformed cells was derived from auto-degradation of the hydroperoxide. Cell lysates originating from 0.1 ml of the culture solution were used for the assay. C: Distribution of the activity in the transformed cells in soluble fraction and membrane fraction obtained from the lysate. D: Immunoblot probed with a polyclonal antibody against bell pepper HPO lyase. Lanes: 1, HPO lyase purified from bell pepper fruits; 2, cell lysate; 3, soluble fraction; 4, membrane fraction. Only the region corresponding to 30–65 kDa is shown.

3.2. Expression of the bell pepper HPO lyase cDNA in E. coli

To verify directly that the cDNA was indeed the HPO lyase, the protein was overexpressed in *E. coli* by use of the expression vector pTrc99A. After addition of an inducer, IPTG, *E. coli* TG1 cells bearing the plasmid were grown at 25°C for 7 h. The crude cell lysate contained detectable enzyme activity to form *n*-hexanal from linoleic acid 13-hydroperoxide (Fig. 2A). With the ΔA234 assay, which follows degradation of the 1-hydroperoxy-2,4-diene moiety in the substrate, the activity could also be detected. During the latter assay, only the chromophore having λ_{max} at 234 nm decreased while no other change could be observed. With immunoblot analysis a polypeptide of about 55 kDa could be detected (Fig. 2D). The mobility of the expressed polypeptide was almost the same as that of the enzyme purified from bell pepper fruits. The heterologously expressed enzyme was essentially detected in the membrane fraction of the cells (Fig. 2C,D).

3.3. Comparison with other P450 sequences

As described above, the bell pepper HPO lyase sequence shares several segments which are highly conserved within the P450 family. These include an ETLR motif in the B domain (modified to ESLR in PL22) and a KDPXXXXPEEF motif in the C domain (modified to PEKF). Although these homologies could assign bell pepper HPO lyase as a member of P450, there also exist unusual but important modifications to the typical P450 consensus sequences. As shown in Fig. 3A, the heme binding cysteine could be allocated to the reasonable site near the C terminus. As many as three residues within the four residues well conserved in P450s are substituted. On the contrary, the region in bell pepper HPO lyase is highly similar to AOSs. It should be noted that, as with AOS, HPO lyase requires neither molecular oxygen nor a reducing equivalent of pyridine nucleotide to exert its activity. These are analogous heme proteins specialized for the metabolism of lipid peroxides [15]. Thus, these conserved differences should have significance to catalyze their specialized activities. In this context, the mammalian enzymes prostacyclin synthase and thromboxane synthase are also the enzymes specialized for lipid peroxides [9,17,18]. Comparison of these sequences with the CYP74 family showed any significant relatedness between them. Song et al. [15] also pointed out that a P450 in a CYP74 family has unprecedented modifications to the typical P450 consensus sequence in the I-helix, which help forming the oxygen-binding pocket. This is also the case with the bell pepper HPO lyase, i.e. a threonine is replaced with isoleucine although glycines are conserved (Fig. 3B). Again, there exists high homology between HPO lyase and AOSs, but the homology with another example of P450 specialized to lipid peroxides, prostacyclin synthase [17], is not so high. Nonetheless, replacement of the threonine residue with isoleucine or alanine can be seen within all the lipid peroxide specific P450s.

Lipid peroxides are compounds which have been extensively investigated because not only the hydroperoxides per se but their degradation products also have important physiological

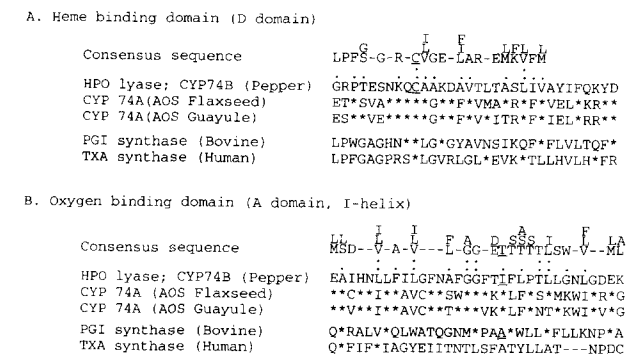


Fig. 3. Comparison of heme binding domain and oxygen binding domain of the bell pepper HPO lyase and P450 consensus sequence, or other P450s specialized for lipid peroxides. The top lines in A and B show consensus sequences highly conserved within the P450 family [14]. Dots and colons beneath the consensus sequence denote residues of the HPO lyase similar and identical to the consensus sequence, respectively. The asterisks indicate residues identical with the HPO lyase sequence. The heme binding cysteine residue in A and the key threonine residue and its replacements are underlined.

effects on living organisms [19]. It is well known that pyrolysis and reaction with iron complexes on lipid peroxides resulted in their decomposition via formation of alkoxy and peroxy radicals [19,20]. A wide variety of products have been identified, and in some reaction conditions, carbon-six aldehydes are formed from n-6 hydroperoxides of fatty acids [20,21]. Reactions of lipid peroxides with the cytochrome P450 family of enzymes have also been intensely investigated. Whereas molecular oxygen and a reduced pyridine nucleotide are required for monooxygenation reactions, these components can be replaced by a lipid peroxide in some cases [22]. It has been established that during such reactions of P450 with lipid peroxides homolysis of the hydroperoxide group takes place [11]. Although the reaction catalyzed by HPO lyase has been thought to proceed via heterolytic scission of the hydroperoxy group [23], the known mechanisms of P450 catalysis and the finding that the lyase is a member of the P450 superfamily have prompted us to re-examine whether or not a radical process is involved. The overexpression system described here will greatly facilitate work in this area.

Acknowledgements: The authors are very grateful to Dr. Kuntz for gifting a bell pepper fruit cDNA library, and to Dr. Nelson for naming the P450 gene subfamily.

References

[1] Vick, B.A. and Zimmerman, D.C. (1976) Plant Physiol. 57, 780–788.
[2] Gardner, H.W. (1991) Biochim. Biophys. Acta 1084, 221–239.
[3] Croft, K.P.C., Jüttner, F. and Slusarenko, A.J. (1993) Plant Physiol. 101, 13–24.
[4] Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T. and Hatanaka, A. (1991) Phytochemistry 30, 2109–2113.
[5] Olías, J.M., Rios, J.J., Valle, M., Zamoraa, R., Sanz, L. C. and Axelrod, B. (1990) J. Agric. Food Chem. 38, 624–630.
[6] Gardner, H.W., Weisleder, D. and Plattner, R.D. (1991) Plant Physiol. 97, 1059–1072.
[7] Shibata, Y., Matsui, K., Kajiwara, T. and Hatanaka, A. (1995) Plant Cell Physiol. 36, 147–156.
[8] Shibata, Y., Matsui, K., Kajiwara, T. and Hatanaka, A. (1995) Biochem. Biophys. Res. Commun. 207, 438–443.
[9] Hecker, M. and Ullrich, V. (1989) J. Biol. Chem. 264, 141–150.
[10] Song, W.-C. and Brash, A.R. (1991) Science 253, 781–784.
[11] White, R.E., Sligar, S.G. and Coon, M.J. (1980) J. Biol. Chem. 255, 11108–11111.
[12] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[13] Kuntz, M., Römer, S., Suire, C., Hugueney, P., Weil, J.H., Schantz, R. and Camara, B. (1992) Plant J. 2, 25–34.
[14] Kalb, V.F. and Loper, J.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7221–7225.
[15] Song, W.-C., Funk, C.D. and Brash, A.R. (1993) Proc. Natl. Acad. Sci. USA 90, 8519–8523.
[16] Pan, Z., Durst, F., Daniele, W.-R., Gardner, H.W., Camara, B., Cornish, K. and Backhaus, R.A. (1995) J. Biol. Chem. 270, 8487–8494.
[17] Hara, S., Miyata, A., Yokoyama, C., Inoue, H., Brugger, R., Lottspeich, F., Ullrich, V. and Tanabe, T. (1994) J. Biol. Chem. 269, 19897–19903.
[18] Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V. and Tanabe, T. (1991) Biochem. Biophys. Res. Commun. 178, 1479–1484.
[19] Halliwell, B. and Gutteridge, J.M.C. (1986) Arch. Biochem. Biophys. 246, 501–514.
[20] Chan, H.W.-S., Prescott, F.A.A. and Swoboda, P.A.T. (1976) J. Am. Chem. Soc. 53, 572–576.
[21] Frankel, E.N., Hu, M.-L. and Tappel, A.L. (1989) Lipids 24, 976–981.
[22] Kadlubar, F.F., Morton, K.C. and Ziegler, D.M. (1973) Biochem. Biophys. Res. Commun. 54, 1255–1261.
[23] Gardner, H.W. and Plattner, R.D. (1984) Lipids 19, 289–293.