Product Specificity in an Entire Series of (ω-6*Z*,ω-9*Z*)-C_{13}~C_{20}-Dienoic Acids and -Dienols for Soybean Lipoxygenase

Akikazu Hatanaka, Tadahiko Kajiwara, Kenji Matsui, and Masanori Yamaguchi

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

Z. Naturforsch. **44c,** 64–70 (1989); received June 27/October 5, 1988

Hydrophobic Interaction, (6*Z*,9*Z*)-Pentadecadienol, Soybean Lipoxygenase, Substrate Specificity

Substrate specificity of soybean lipoxygenase-1 (EC 1.13.11.12) was studied using as synthetic substrate analogs an entire series of (ω-6*Z*,ω-9*Z*)-C_{13}~C_{20}-dienoic acids and (ω-6*Z*,ω-9*Z*)-C_{12}~C_{20}-dienols. The relative activity of lipoxygenase-1 against linoleic acid ($C_{18:2}$COOH) was the highest among the dienoic acids and that of (6*Z*,9*Z*)-pentadecadienol ($C_{15:2}$OH) was the highest among the dienols. Soybean lipoxygenase-1 oxygenated almost at ω-6 position of the dienoic acids independently of the chain length. With the dienols as substrate, the position of oxygenation was much influenced by the chain length. Above all, $C_{15:2}$OH was selectively oxygenated at ω-10 rather than at ω-6. Kinetic analyses revealed that longer and more hydrophobic dienols had higher affinity but lower velocity of oxygenation reaction. But higher velocity was obtained when smaller K_m value was observed for the dienoic acids.

To elucidate stereospecificity of the oxygenation to $C_{15:2}$OH, optically active authentic samples were prepared *via* biological asymmetric reduction with baker's yeast. The ω-6 oxygenation to $C_{15:2}$OH was found to be (*S*)-specific (*R*/*S* = 26/74) as is that to $C_{18:2}$COOH, whereas the ω-10 oxygenation showed low enantioselectivity (*R*/*S* = 60/40).

Introduction

Lipoxygenase-1 (EC 1.13.11.12) of soybean catalyzes the oxygenation of the (*Z*,*Z*)-1,4-pentadiene system of unsaturated fatty acids and specifically produces 13-L-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid from linoleic acid. The native enzyme contains an Fe(II) (non heme), which is converted to Fe(III) by product stoichiometrically to give the catalytically active form of the enzyme [1]. Recently, Shibata *et al.* [2] determined the primary structure of lipoxygenase-1.

Abbreviations: $C_{12:2}$OH, (3*Z*,6*Z*)-dodecadienol; $C_{13:2}$OH, (4*Z*,7*Z*)-tridecadienol; $C_{14:2}$OH, (5*Z*,8*Z*)-tetradecadienol; $C_{15:2}$OH, (6*Z*,9*Z*)-pentadecadienol; $C_{16:2}$OH, (7*Z*,10*Z*)-hexadecadienol; $C_{17:2}$OH, (8*Z*,11*Z*)-heptadecadienol; $C_{18:2}$OH, linoleyl alcohol; $C_{19:2}$OH, (10*Z*,13*Z*)-nonadecadienol; $C_{20:2}$OH, (11*Z*,14*Z*)-eicosadienol; $C_{13:2}$COOH, (4*Z*,7*Z*)-tridecadienoic acid; $C_{14:2}$COOH, (5*Z*,8*Z*)-tetradecadienoic acid; $C_{15:2}$COOH, (6*Z*,9*Z*)-pentadecadienoic acid; $C_{16:2}$COOH, (7*Z*,10*Z*)-hexadecadienoic acid; $C_{17:2}$COOH, (8*Z*,11*Z*)-heptadecadienoic acid; $C_{18:2}$COOH, linoleic acid; $C_{19:2}$COOH, (10*Z*,13*Z*)-nonadecadienoic acid; $C_{20:2}$COOH, (11*Z*,14*Z*)-eicosadienoic acid; (*S*)-MTPA-Cl, (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid chloride; HPLC, high performance liquid chromatography; L-1, soybean lipoxygenase-1.

Reprint requests to Dr. T. Kajiwara.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0100–0064 $ 01.30/0

Lipoxygenase is widespread in nature. In animal tissue, three major lipoxygenases were discovered, *i.e.*, arachidonic acid 12-lipoxygenase [3], 5-lipoxygenase [4], and 15-lipoxygenase [5]. They convert arachidonic acid to metabolites such as prostaglandin, leukotriene, *etc.*, which have various important physiological roles. In plant tissues, two major lipoxygenases were discovered, *i.e.*, linolenic acid 9-lipoxygenase [6], and 13-lipoxygenase [7] which convert linolenic acid to fragrance compounds such as jasmonic acid [8, 9] and hexenals [10]. The former is known to have hormonal effects on plants [11] and the latter is known to be an allelo-chemical [12].

The hydrophobic interaction between substrate and lipoxygenase is thought to be one of the factors controlling regiospecificity of oxygenation to a pentadiene system, but a precise mechanism has not been elucidated yet. Substrate specificity for natural substrates could offer limited information about the hydrophobic interaction between enzyme and substrate. Therefore, it is needed to study substrate-activity profile using synthetic substrate analogous to linoleic acid.

In this study, as synthetic analogs, a series of (ω-6*Z*,ω-9*Z*)-C_{13}~C_{20}-dienoic acids and (ω-6*Z*,ω-9*Z*)-C_{12}~C_{20}-dienols was used as substrates for soybean lipoxygenase-1.

Materials and Methods

Materials

Two different preparations of soybean lipoxygenase-1 were used for the experiments: type-I and type-V (Sigma Chemical Co., St. Louis, Mo.). Further purification of the type-I lipoxygenase was done by chromatography on DEAE Sephadex A-50 [13] (Pharmacia Fine Chemicals, Uppsala, Sweden). Linoleic acid and (10*Z*,12*Z*)-eicosadienoic acid which were a gift from Nippon Oil and Fats Co. were >98% purity grade, and the corresponding alcohols were prepared by reduction of the acids with lithium aluminium hydride.

Synthesis of substrates

Aliphatic C_{13}~C_{20} substrates containing a (ω-6*Z*,ω-9*Z*)-pentadiene system were synthesized by the Wittig coupling [14] between C_9-phosphonium salt and Cn-aldehyde (n = 3~8, 10). (10*Z*,13*Z*)-Nonadecadienoic acid, (8*Z*,11*Z*)-heptadecadienoic acid, and (7*Z*,10Z)-hexadecadienoic acid, and (5*Z*,8*Z*)-tetradecadienoic acid, and (4*Z*,7*Z*)-tridecadienoic acid were synthesized *via* the corresponding (ω-6*Z*,ω-9*Z*)-dienols according to the same procedure described for (6*Z*,9*Z*)-pentadecadienoic acid. (3*Z*,6*Z*)-Dodecadienol was synthesized as previously reported [15]. The synthetic specimens were used as substrates having >95% purity after purification by column chromatography.

Preparation of (6Z,9Z)-pentadecadienoic acid

(3*Z*)-Nonenyltriphenylphosphonium iodide (C_9 counterpart) was prepared according to Cohen *et al.* [14]. The C_6 counterpart, (2-tetrahydropyranyloxy)-hexanal, was prepared by oxidation of monopyranyl-ether-hexanol. The (*Z*)-selective Wittig reaction [14, 16] between (2-tetrahydropyranyloxy)-hexanal and (3*Z*)-nonenyltriphenylphosphonium iodide in HMPA-THF solvent at −78 °C using butyl−lithium as a base gave a (6*Z*,9*Z*)-diene pyranylether in 65% yield, >95% purity. After removal of the protecting group in H_3PO_4−MeOH, the crude alcohol was purified by silica gel (Woelm Pharma, W. Germany) column chromatography (eluted with ether and hexane) to give (6*Z*,9*Z*)-pentadecadienol. The purified alcohol was converted to (6*Z*,9*Z*)-pentadecadienoic acid by Jones oxidation. The structure of (6*Z*,9*Z*)-pentadecadienoic acid was substantiated by ^{13}C NMR and IR analyses: ^{13}C NMR ($CDCl_3$, ppm) 180.3 (COOH), 130.4 (CH), 129.3 (CH), 128.7 (CH), 127.8 (CH), 34.1 (CH_2), 31.6 (CH_2), 29.4 (CH_2), 29.1 (CH_2), 27.3 (CH_2), 26.9 (CH_2), 25.7 (CH_2), 24.4 (CH_2), 22.6 (CH_2), 14.1 (CH_3); IR (KBr, cm^{-1}) 3400−2500 (COOH), 3015 (C=C−H), 2960, 2925, 2860 (C−H), 1715 (C=O), 1650 (C=C), 920 (C=O), 710 (*Z*, C=C−H).

Assay of lipoxygenase activity

Lipoxygenase activity was determined polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co.) or spectrophotometrically by monitoring an increase in absorbance at 234 nm at 25 °C. A standard reaction mixture (3 ml) containing 50 mM borate buffer (pH 9.0), enzyme solution (0.1 ml, 0.72 units) and substrate (1 μmol dissolved in 0.2% Tween 20, final concentration, 0.67 mM). One unit of enzyme was expressed as the amount of enzyme consuming 1 μmol O_2/min at 25 °C.

Preparation of lipoxygenase reaction products

Soybean lipoxygenase (800 units) was added to a reaction mixture (60 ml) consisting of 50 mM borate buffer (pH 9.0) and 6 ml of substrate solution (10 mg dissolved in 0.2% Tween 20) in a 200 ml sealed flask. The complete reaction mixture was incubated for 90 min at 15 °C with gentle stirring under O_2 (1.5 kg/cm^2). The reaction mixture was acidified with 2 N HCl (except for the dienols as substrate) and then extracted with 250 ml of ether−methanol (4:1, v/v). After the extract was evaporated *in vacuo*, the residue was passed through a short silica gel column (∅ 10 × 30 mm) with ether. Evaporation of the eluate gave crude hydroperoxides. The hydroperoxides were reduced with $NaBH_4$ in 4 ml of methanol−borate buffer (1:1, v/v, pH 9.0). The isolated reduced compound dissolved ether was converted by treatment with diazomethane to a hydroxy-ester or a diol (from alcohol) isomers.

Preparation of MTPA ester derivative of enzyme reaction products

MTPA-Cl ((*S*)-α-methoxy-α-trifluoromethylphenylacetic acid chloride, 900 mg) was added to the enzyme reaction product, *e.g.* hydroxypentadecadienol (**1**) (90 mg) in 15 ml of CCl_4−pyridine (2:1, v/v) [17]. After 12 h stirring at room temperature, the reaction mixture was extracted with ether. The ether extract

was concentrated *in vacuo* to give a di-MTPA ester derivative (**2**). Ozonolysis of the di-MTPA ester derivative was performed in 25 ml of dry methanol–CH_2Cl_2 (3:2, v/v) at −76 °C for 15 min and subsequently the ozonide was treated with peracetic acid. The products were esterified with diazomethane to give a α-MTPA ester-methyl ester derivative (**7**, **8**).

*Preparation of authentic α-MTPA ester-methyl esters of 2-(R)-7-dihydroxy-heptanoate (R-**5**) and 2-(S)-hydroxy-heptanoate (S-**6**)*

The authentic MTPA ester derivatives of methyl 2,7-dihydroxy-heptanoate (rac.-**5**) and methyl 2-hydroxy-heptanoate (rac.-**6**) were synthesized by α-hydroxylation of methyl 7-(2-tetrahydropyranyloxy)-heptanoate and methyl heptanoate, respectively, using lithium diisopropylamide. The racemic 2-hydroxy-methyl esters (rac.-**5**, **6**) were oxidized to α-keto methyl esters (**3**, **4**) by $KMnO_4$–$MgSO_4$ in a H_2O-hexane solvent system. Subsequently the keto-esters were reduced using baker's yeast as previously reported [18] to give methyl 2-(*R*)-hydroxy-7-(2-tetrahydropyranyloxy)-heptanoate and methyl 2-(*S*)-hydroxy-heptanoate (*S*-**6**), respectively. After methanolysis of the pyranylether, methyl 2-(*R*)-7-dihydroxy-heptanoate (*R*-**5**) and methyl 2-(*S*)-hydroxy-heptanoate were converted to the corresponding MTPA esters (*R*-**7**, *S*-**8**), respectively. The configuration of methyl 2-(*R*)-7-dihydroxy-heptanoate was assigned by correlation with the MTPA ester of methyl 2-(*R*)-10-dihydroxy-decanoate derived from 2-(*R*)-hydroxy-10-undecenoate [19]. The optically active methyl 2-hydroxy-heptanoate was identical with MTPA ester derivative (*S*-**8**) of 2-(*S*)-hydroxy-heptanoate from 13-L-hydroperoxylinoleic acid [20].

Determination of positional, geometrical, and optical isomer compositions of enzymic reaction products by HPLC analysis

HPLC analysis was performed with a Shimadzu Dupont liquid chromatograph (LC-5) equipped with a Zorbax-SIL column (∅ 4.6 × 25 cm; *n*-hexane–ethanol 99.5/0.5, v/v, flow rate 2.0 ml/min for methyl hydroxy–dienoic acids from carboxylic acid, 98/2, v/v, flow rate 2.0 ml/min for hydroxy–dienols from alcohol, 99.5/0.5, v/v, flow rate 1.0 ml/min for MTPA derivatives) with UV detection at 262 nm and 233 nm.

Results

Fig. 1. shows the relative activity and the product specificity of soybean lipoxygenase-1 (L-1) for substrates of different chain length from (ω-6*Z*,ω-9*Z*)-tridecadienoic acid to (ω-6*Z*,ω-9*Z*)-eicosadienoic acid, and the corresponding alcohols including (ω-6*Z*,ω-9*Z*)-dodecadienol. As expected, linoleic acid ($C_{18:2}$COOH, natural substrate) was the best substrate among the acids (Fig. 1, left). With either

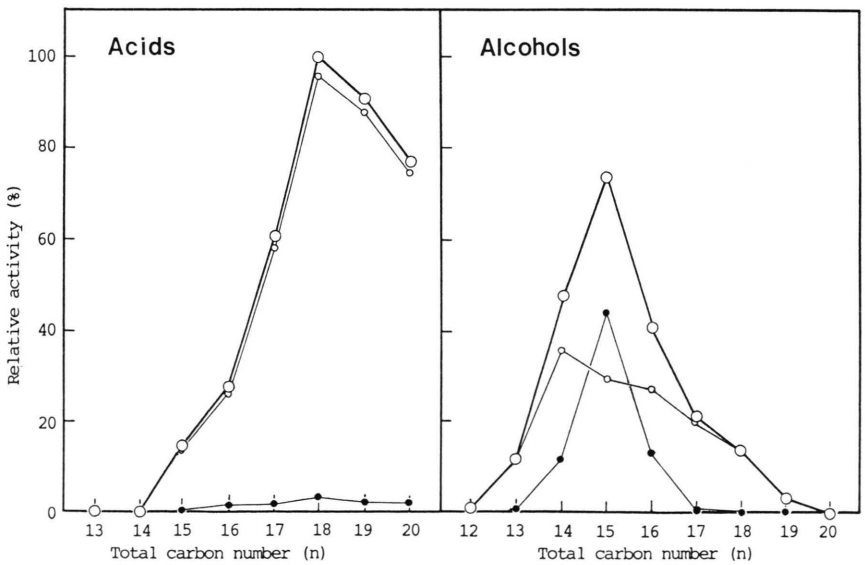


Fig. 1. Relative activity (○——○) of L-1 for synthetic substrates were plotted against total carbon number (n). Relative ω-6 oxygenation activity (○——○) and ω-10 oxygenation activity (●——●) were calculated according to the HPLC analyses of the products derived from respective substrates.

longer or shorter substrates, the oxygenation activity decreased, and O_2 consumption was not detected for $C_{13:2}$COOH. The (ω-6*Z*,ω-9*Z*)-dienols could also act as substrate (Fig. 1, right). A particular observation was that L-1 showed highest activity for $C_{15:2}$OH ((6*Z*,9*Z*)-pentadecadienol) among alcohols, and this activity reached 76% of that for $C_{18:2}$COOH.

All the substrates used here have two possible oxygenation sites (ω-6 and ω-9 position). To investigate the positional selectivity in the oxygenation, products formed during incubation of the dienoic acids and dienols with L-1 were quantitatively analyzed by HPLC. The HPLC analyses showed that all (*E*)-isomers was scarcely formed during incubations. As is well known, L-1 oxygenates at ω-6 position of linoleic acid. This is also the case for the synthetic dienoic acids, and the regioselectivity was nearly >90% for all acids. With the dienols, however, the regioisomer ratio of lipoxygenase products was substantially changed among the substrates used. The oxygenating activity at ω-10 position was the highest for $C_{15:2}$OH (see Table I) and the activity at ω-6 position (%) was the highest for $C_{14:2}$OH. Higher activity at ω-10 than that at ω-6 was observed only for $C_{15:2}$OH, with the other dienols the activity at ω-6 was more or less higher than that at ω-10.

Kinetic parameters of L-1 with synthetic substrates which had a significant activity were determined (Fig. 2). Surprisingly, a series of kinetic parameter observed with the dienoic acids and dienols showed very different profile. The plots for carboxylic acids (Fig. 2, left) indicated that the acid which have higher K_m value had lower V_{max}. In contrast, those for alcohols (Fig. 2, right) indicated that the substrate which had higher K_m value had higher V_{max} except for $C_{14:2}$OH. This dienol showed almost the same tendency for $C_{15:2}$OH.

The enantiospecificity of oxygenation to $C_{15:2}$OH was determined by HPLC analysis of MTPA ester derivatives of the product. Authentic optically active MTPA derivatives were prepared by biologically asymmetric reduction using baker's yeast as described in Materials and Methods. As shown in Fig. 3, each MTPA ester derivative was coincident with authentic specimens, and the predominant absolute configuration was determined as (*S*) configuration for the ω-6 oxygenation activity to $C_{15:2}$OH. This was identical with the oxygenation at ω-6 in

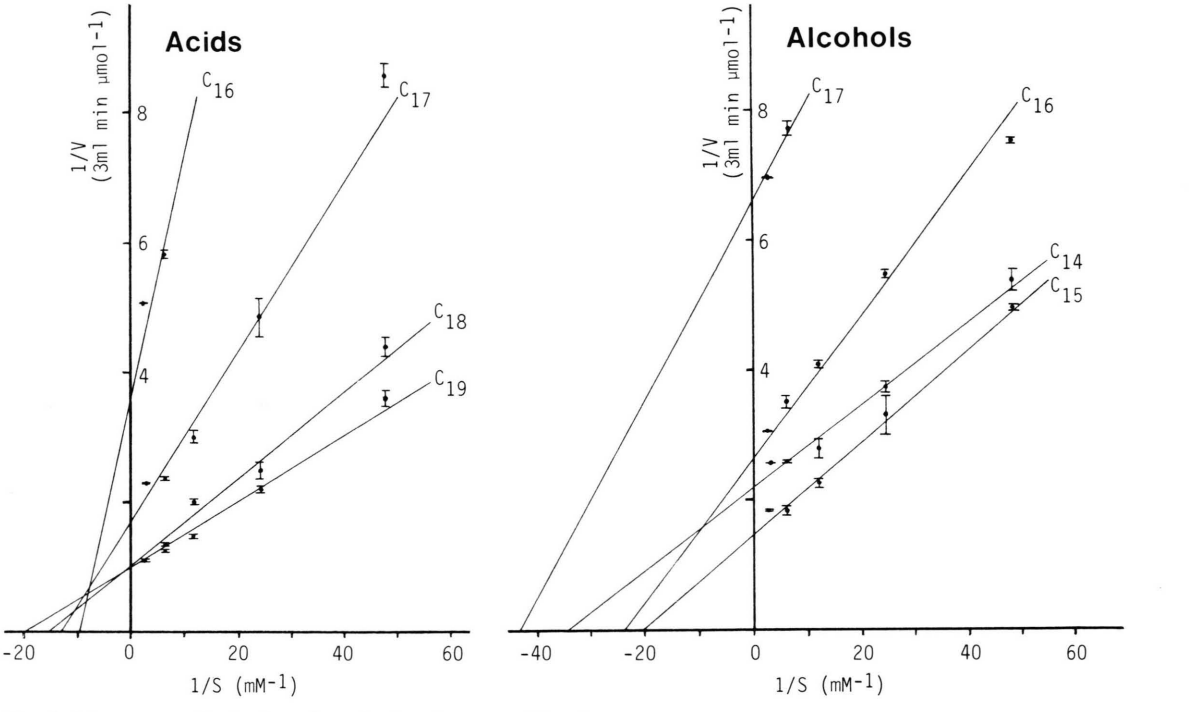


Fig. 2. Lineweaver-Burk plot of synthetic substrates. Kinetic parameters were calculated by the least square method with Taylor expansion [21]. To emphasis the differences in kinetic parameter some dots were excluded.

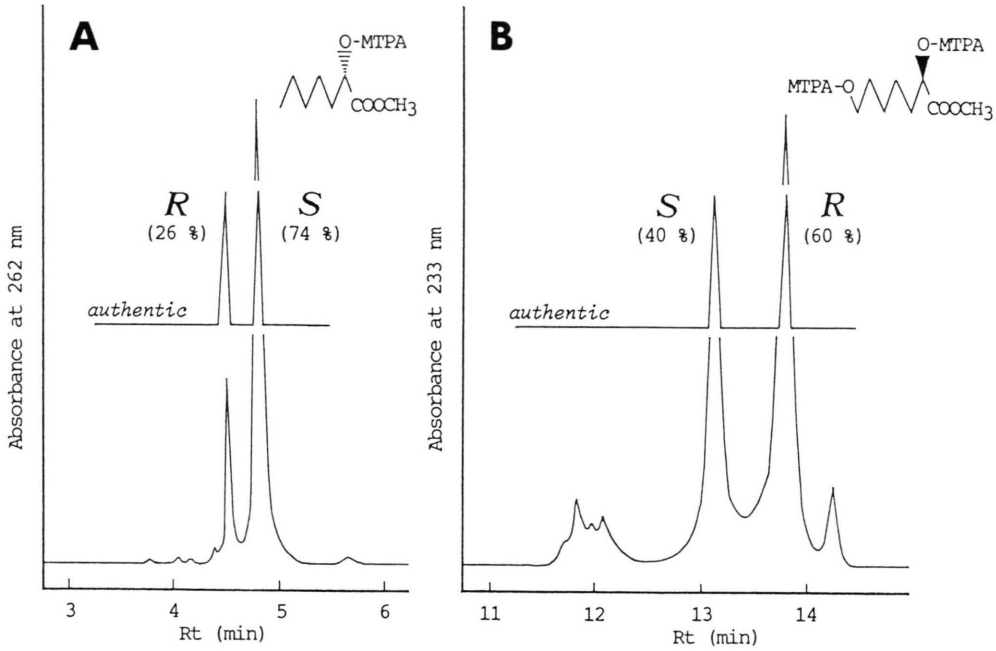
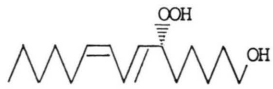
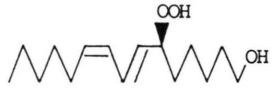


Fig. 3. HPLC analyses of MTPA derivatives of products after incubation of (ω-6*Z*,ω-9*Z*)-pentadecadienol with L-1. The enantiomeric compositions for ω-6 oxygenation activity (panel A) and ω-10 oxygenation activity (panel B) were calculated from peak areas.

linoleic acid. But inverse preference in enantioselectivity was evident for the ω-10 oxygenation activity although lower selectivity was observed.

The stereo- and regioselectivity of L-1 for $C_{15:2}OH$ are summarized in Table I. As seen in the table, $C_{15:2}OH$ was converted into four major isomers by

Table I. Summary of product specificity of L-1 for (ω-6Z,ω-9Z)-pentadecadienol.

Product	Ratio (%)	Regio isomer (%)
 ω-6S-HPO	24.4	33.0
 ω-6R-HPO	8.6	
 ω-10R-HPO	40.2	67.0
 ω-10S-HPO	26.8	

L-1. ω-6-(*S*), ω-6-(*R*), ω-10-(*R*), and ω-10-(*S*) hydroperoxides were produced at ratios of 24.4%, 8.6%, 40.2%, and 26.8%, respectively.

Discussion

In this study, series of (ω-6*Z*,ω-9*Z*)-dienoic acids and (ω-6*Z*,ω-9*Z*)-dienols were synthesized to elucidate the substrate recognition determining product specificity of L-1.

With the dienoic acids, changes of the chain length from the terminal group to the pentadiene system scarcely affected the regioselectivity of products although decreased relative activity. This suggested that L-1 recognizes the chain length from the (*Z*,*Z*)-pentadiene system to the carboxy group in the substrate and that the region recognizing this part of the substrate is a hydrophobic one. The hydrophobic pocket of L-1 was previously suggested by inhibitory experiments using several straight chain alcohols [22].

Unsaturated fatty alcohols were used as substrate analogs having no negative charge but the net hydrophobicity was higher than that of the corresponding dienoic acids. Unexpectedly, $C_{15:2}$OH was oxygenated best of all the other dienols and both shorter and longer alcohols including linoleyl alcohol were less oxygenated. The affinity of alcoholic substrates estimated from K_m values tended to increase with a decrease of V_{max}. From these results, it was suggested that the decrease of the oxygenation activity was caused by higher hydrophobicity of longer alcohols which might bind with the hydrophobic region of L-1 too tightly to allow rapid dissociation of products. The regioselectivity of oxygenation to fatty alcohols suggested inverse spatial arrangement of this alcohol. The structural unit ((ω-6*Z*,ω-9*Z*)-pentadecadiene) of $C_{15:2}$OH has a plane symmetry at diallyl carbon. The hydrophobic pocket of L-1 where ω-terminal of linoleic acid would bind might accept hydrophobicity consisting of five methylene groups. The longer or shorter dienols are thought to prefer binding at normal arrangement, which might result in a decrease of ω-10 oxygenation activity. The inverse spatial arrangement of a substrate with L-1 was reported in case of the double dioxygenation of arachidonic acid [23]. In that case steric structure of the second oxygenation was coincident with the concept of inverse spatial arrangement. But, the steric structure of ω-10 oxygenated $C_{15:2}$OH was (*R*) rather than (*S*) although the selectivity was not so high. This contradiction could not be explained yet, but ambiguity of enzyme-substrate interaction might contribute this.

The comparison of the ω-6 oxygenation activity of the dienols and dienoic acids with various chain length revealed the existence of a carboxy group recognizing site in L-1 which may offer ionic interaction with the carboxy group. The strong interaction of substrate with this site would prohibit diallyl hydrogen contact with the reaction center of L-1 if the dienoic acids shorter than C_{18} were applied. Because hydroxy group of the dienols would interact weaker with this site, diallyl hydrogen could interact with the reaction center easier.

Our results suggest a substrate recognition by L-1 as follows, 1) the reaction center is surrounded by a strong hydrophobic region which excludes polar groups, 2) terminal carboxy groups have an important role in determining product specificity, and exchange of the terminal carboxy group by a hydroxy group reduced this specificity.

[1] J. F. G. Vliegenthart and G. A. Veldink, in: Free Radicals in Biology (W. A. Pryor, ed.) **5,** p. 29, Academic Press, New York.
[2] D. Shibata, J. Steczko, J. E. Dixon, M. Hermodson, R. Yazdanparast and B. Axelrod, J. Biol. Chem. **262,** 10080 (1987).
[3] M. Hamgerg and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A. **71,** 3400 (1974).
[4] P. Borgeat, M. Hamgerg, and B. Samuelsson, J. Biol. Chem. **251,** 7816 (1976).
[5] P. Borgeat and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A. **76,** 2148 (1979).
[6] T. Galliard and D. R. Phillips, Biochem. J. **124,** 431 (1971).
[7] J. P. Christopher, E. K. Pistorius, and B. Axelrod, Biochim. Biophys. Acta **284,** 54 (1972).
[8] B. A. Vick and D. C. Zimmerman, Plant Physiol. **67,** 92 (1981).
[9] B. A. Vick and D. C. Zimmerman, Plant Physiol. **80,** 202 (1986).
[10] J. Sekiya, S. Numa, T. Kajiwara, and A. Hatanaka, Agric. Biol. Chem. **40,** 185 (1976).
[11] B. A. Vick and D. C. Zimmerman, Plant Physiol. **69,** 1103 (1982).
[12] L. M. Riddiford, Science **158,** 139 (1967).
[13] B. Axelrod, T. M. Cheesbrough, and S. Laakso, Methods Enzymol. **71,** 441 (1981).
[14] N. Cohen, B. L. Banner, R. J. Lopesti, F. Wong, M. Rosenberger, Yu-Y. Liu, E. Thom, and A. A. Liebman, J. Am. Chem. Sci. **105,** 3661 (1983).
[15] T. Kajiwara, J. Sekiya, Y. Odake, and A. Hatanaka, Agric. Biol. Chem. **41,** 1481 (1977).
[16] T. Kajiwara, H. Yoshikawa, T. Saruwatari, A. Hatanaka, T. Kawai, M. Ishihara, and T. Tsuneya, Phytochemistry **27,** 1643 (1988).
[17] J. A. Dale, D. L. Dull, and H. S. Mosher, J. Org. Chem. **34,** 2543 (1969).
[18] S. Tsuboi, H. Furutani, and A. Tanaka, Bull. Chem. Soc. Jpn. **60,** 833 (1987).
[19] S. Yamaguchi, Tetrahedron Lett. **1,** 89 (1977).
[20] A. Hatanaka, T. Kajiwara, J. Sekiya, and M. Asano, Z. Naturforsch. **39c,** 171 (1984).
[21] M. Sakoda and K. Hiromi, J. Biochem. **80,** 547 (1976).
[22] H. Mitsuda, K. Yasumoto, and A. Yamamoto, Arch. Biochem. Biophys. **118,** 664 (1967).
[23] C. P. A. Van Os, G. P. M. Rijke-Schilder, H. V. Halbeek, J. Verhagen, and J. F. G. Vliegenthart, Biochim. Biophys. Acta **663,** 177 (1981).