Biosynthesis of Leaf Alcohol:
Kinetic Study of Lipoxxygenase
Activation Process Caused by
Hydroperoxylinoleic Acid

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The authors have been studying the biosynthetic mechanisms of leaf alcohol (3Z-hexenol: I) and cucumber alcohol (2E, 6Z-nonadienol: II). In the previous paper,¹⁾ the biosynthetic pathway of I *via* 13-peroxide from linolenic acid (III), has been proposed. On the other hand, II is biosynthesized *via* peroxide whose oxygen is introduced into carbon-9 in III.²⁾

The authors speculate that these specific oxygenation of the unsaturated fatty acid in the biosynthetic pathway may be catalyzed by some lipoxxygenase-like enzyme in looking for the enzyme system responsible for the production of peroxides from III and linoleic acid (IV) under aerobic conditions.³⁾ In this connection, the authors have studied the mechanism of lipoxxygenase reaction in detail using soybean lipoxxygenase-I and -II.⁴⁾ The product 13-L-hydroperoxylinoleic acid (V) plays a peculiar role in the lipoxxygenase reaction, since the induction period, which is observed when soybean lipoxxygenase-I is incubated with linoleic acid, can only be abolished by addition of V,⁵⁾ whereas 9-D-hydroperoxylinoleic acid is ineffective. Groot *et al.* have reported that the product converts ferrous state (E-Fe²⁺) in active site to ferric state (E-Fe³⁺) by ESR measurement at 15 K.⁶⁾ Finazzi-Agro' *et al.* have found that the fluorescence of the enzyme centered around 328 nm

on excitation at 280 nm is quenched by replacing oxygen in the solution by argon, and that a further quenching is observed by addition of V.⁷⁾ They have investigated the mechanism of this fluorescence by using external perturbing agents like KI, imidazole, NaN₃, *etc.*⁸⁾ and suggested that the change in fluorescence is related with the interconversion of ferrous state of iron in the native enzyme into the ferric state.

In this paper, the kinetics of the interaction between lipoxxygenase and V was studied by the fluorescence stoppedflow method.⁹⁾ Dependence of the reciprocal relaxation time (1/ τ) on the concentration suggested a two-step mechanism in which a fast bimolecular association is followed by a slow unimolecular process.

Lipoxxygenase-I was purchased from Sigma Co., Ltd. St. Louis and was purified by chromatography on DEAE-Sephadex A-50.¹⁰⁾ Disc electrophoresis on polyacrylamide gel showed the enzyme as one distinct band. The enzyme concentration was estimated from the absorption at 280 nm [$E_{280}^{0.1\%}=1.43$].¹¹⁾

According to the procedure of Hatanaka *et al.*, V was prepared and purified.^{4,12)} All reagents used for stopped-flow measurement were dissolved in 0.1 M ammonium-ammonium chloride buffer at pH 9.0 containing 5% ethanol, which was added to dissolve V.

A stopped-flow apparatus (Union Giken SF 70) was used for the kinetic study.⁹⁾ The fluorescence emission was observed through a cut-off filter (transmitting above 300 nm) at right angle to the excitation beam (280 nm) from a D₂ lamp (200 W). The reaction signals were averaged for eight runs with a 10-channel digital memory equipment (Union Giken HD-100) and displayed with a transient memory and a X-Y recorder. A typical example of the trace of the fluorescence change was observed after mixing lipoxxygenase (5.5×10^{-7} M) and V (1.38×10^{-6} M) by the stopped-flow method at pH 9.0 and 10.2°C.

The change in fluorescence intensity actually observed with the stopped-flow method was the same to the total change observed with the

FIG. 1. Dependence of Reciprocal Relaxation Time ($1/\tau$) on $(P)_0 - (E)_0$ at Various Temperature.

$(P)_0$ and $(E)_0$ designate the initial concentrations of product and enzyme, respectively. $[(P)_0 - (E)_0]$ is well approximated to the sum of equilibrium concentration of product and enzyme in this system. The dashed lines are theoretical curves using eq. (3) and the values listed in Table I. I, lipoxxygenase; $5.5 \times 10^{-7} \sim 1.65 \times 10^{-6}$ M.

●, 5.0°C; ○, 10.2°C; ○, 15.2°C; ⊕, 25.0°C.

static measurement. The time course followed the pseudo-first order kinetics giving a single relaxation time. Figure 1 shows the dependence of the reciprocal relaxation time ($1/\tau$) on the concentration of the free V $[(P)_0 - (E)_0]$ at various temperatures. The reciprocal relaxation time ($1/\tau$) increased with the free product concentration, tend to saturate at higher concentrations. These results suggest a two-step mechanism shown below;

where E and P are the enzyme and the product, respectively. P' is unknown reaction product of V.⁶⁾ If the rate constants, k_1 and k_{-1} of the bimolecular association process, are much larger than that of the unimolecular process, k_2 and k_{-2} , two relaxation times, τ_1 and τ_2 exist ($\tau_1 < \tau_2$) as follows,¹³⁾

$$1/\tau_1 = k_1(\bar{C}_E + \bar{C}_P) + k_{-1} \quad (2)$$

$$1/\tau_2 = k_{-2} + k_1 k_2 / k_{-1} (\bar{C}_E + \bar{C}_P) / [1 + k_1 / k_{-1} (\bar{C}_E + \bar{C}_P)] \quad (3)$$

where $\bar{C}_E$ and $\bar{C}_P$ are the equilibrium con-

TABLE I. VALUES OF THE RATE PARAMETERS AT VARIOUS TEMPERATURES OBTAINED BY THE LEAST SQUARE METHOD USING EQ. (3)

Temperature (°C)	k_{-1}/k_1 (M)	k_2 (sec ⁻¹)
5.0	2.82×10^{-5}	110
10.2	1.04×10^{-5}	100
15.2	1.24×10^{-5}	169
25.0	0.805×10^{-5}	175

centrations of E and P , respectively. The result shown in Fig. 1 is consistent with equation 3; at low concentration of product, reciprocal relaxation time is linear in concentration of the free product ($\bar{C}_E + \bar{C}_P \approx (P)_0 - (E)_0$) and it becomes constant at high concentrations. The vertical intercept in Fig. 1 is quite close to zero, which suggests $k_{-2} \approx 0$. The values of k_{-1}/k_1 and k_2 obtained by the least square method using eq. (3) are shown in Table I. The dissociation constant of the product with the enzyme, k_{-1}/k_1 , (about 10^{-5} M) is rather small compared with the Michaelis constant K_m of IV (about 10^{-3} M). Therefore, if the substrate and the product interact at the same site, a remarkable product inhibition should be

observed, but it is not the case for lipoxygenase reaction. Thus it may be suggested that the substrate and the product interact at different sites of lipoxygenase as was suggested by Smith *et al.*⁵⁾ Another possibility would be that the active enzyme ($E-Fe^{3+}$) has less association constant toward product than inactive enzyme ($E-Fe^{3+}$). The slow unimolecular step may be the rate-limiting step of product activation. The strong dependences of k_{-1}/k_1 and k_2 on temperature were not observed (Table I), hence the accurate estimation of activation parameters was unsuccessful. More detailed kinetic investigation on the activation-process of lipoxygenase are in progress.

REFERENCES

- 1) A. Hatanaka and T. Harada, *Phytochem.*, **12**, 2341 (1973).
- 2) A. Hatanaka, T. Kajiware and T. Harada, *Phytochem.*, **14**, 2242 (1975).
- 3) J. Sekiya, S. Numa, T. Kajiware and A. Hatanaka, *Agr. Biol. Chem.*, **40**, No. 1 (1976).
- 4) A. Hatanaka *et al.*, unpublished.
- 5) W. L. Smith and W. E. M. Lands, *J. Biol. Chem.*, **247**, 1038 (1972).
- 6) J. J. M. C. Groot, G. A. Veldink, J. F. G. Vliegenthart, J. Boldingh, R. Wever and B. F. V. Gelder, *Biochim. Biophys. Acta*, **377**, 71 (1975).
- 7) A. Finazzi-Agro, L. Avigliano, G. A. Veldink, J. F. G. Vilegenthart and J. Boldingh, *Biochim. Biophys. Acta*, **326**, 462 (1973).
- 8) A. Finazzi-Agró, L. Avigliano, M. R. Egmond, G. A. Veldink and F. G. Vliegenthart, *FEBS LETTERS*, **52**, 73 (1975).
- 9) H. Nakatani and K. Hiromi, *J. Biochem.*, **76**, 1343 (1974).
- 10) J. Christopher, E. Pistorius and B. Axelrod, *Biochim. Biophys. Acta*, **198**, 12 (1970).
- 11) M. Roza and A. Franke, *Biochim. Biophys. Acta*, **326**, 279 (1973).
- 12) A. L. Tappel, P. D. Boyer and W. O. Lundberg, *J. Biol. Chem.*, **199**, 267 (1952).
- 13) "New Method of Biophysical Study," Vol. 2, ed. by K. Hiromi, Kagakudojin, Tokyo, Japan, 1967, p. 154.