

# Effect of Modification of Arginine Residues on the Activity of Soybean Lipoygenase-1

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Arginine residues of soybean lipoygenase-1 was modified with an arginine-directed chemical modifier, 2,3-butanedione. Although inactivation was not visible if the enzyme reaction was monitored under the standard assay condition (83.3  $\mu$ M linoleic acid dispersed in 200 mM sodium borate, pH 9.0), rapid inactivation was observed with 5 mM sodium borate, pH 8.0. The inactivation was protected by the addition of a substrate, linoleic acid, in the modification mixture. Kinetic analyses indicated that one arginine residue accounted for the inactivation.

Enzymological analyses showed that the modification narrowed the pH-activity profile of L-1 and made L-1 sensitive to salt concentration of the assay solution. Strong inactivation by modification was found at low salt concentration and low pH. This was not due to a physical change of the linoleic acid. On the other hand, product specificity of L-1 was not altered after modification. Taken together, the modified arginine residue(s) was thought to be not essential to the catalysis but have an important role in supporting an ideal electrostatic interaction within L-1 and/or between L-1 and a substrate even in sub-optimal reaction conditions.

## Introduction

Lipoygenase (EC 1.13.11.12) is a nonheme iron dioxygenase that plays a major role in polyunsaturated fatty acid metabolism in plants and animals (Yamamoto, 1992; Siedow, 1991; Vick and Zimmerman, 1987). Enzymatic properties of a lipoygenase isolated from soybean seeds, lipoygenase-1 (L-1), have been extensively investigated because it is readily obtained abundantly in a purified form. From linoleic acid L-1 abstracts pro-S hydrogen bound to a methylene carbon atom ( $C_{11}$ ) of the pentadiene system and introduces dioxygen at  $C_{13}$  in the *S* configuration to yield 13S-hydroperoxy-(9Z,11E)-octadecadienoic acid (Vick and Zimmerman, 1987). From 1987, when Shibata *et al.* (1987) firstly reported the primary structure of L-1, reports on cDNA sequences of lipoygenase in plants and animals have been accumulating (Shibata *et al.*, 1988; Yenofsky *et al.*, 1988; Ealing and Casey, 1988; Sigal *et al.*, 1988; Yoshimoto *et al.*, 1990; Ohta *et al.*, 1992; Melan *et al.*, 1993; Melan *et al.*, 1994). Furthermore, Boyington

*et al.* (1993) reported the three-dimensional structure of L-1. With this improvement, several amino residues in L-1 have been postulated to have an essential role in catalysis and substrate binding. Although essential roles of some histidine residues were confirmed with site-directed mutagenesis (Steczko *et al.*, 1992), those of the other residues have not been examined in detail. At first, we intended to examine a role of arginine residues in L-1 with chemical modification. L-1 has an alkaline pH optimum (ranging from 8 to 10) where the terminal carboxyl group of a fatty acid substrate ionized to the carboxylate anion. Gardner (1989) reported that the carboxylate anion of a substrate is important for L-1 to recognize it in a proper orientation in the reaction center, and that recognition of the anion has a crucial role in determining product specificity of L-1. In many enzymes, an arginine residue has been reported to function as a residue recognizing a carboxylate anion of a substrate (Bateman *et al.*, 1989; Katamori *et al.*, 1990). On the other hand, comparison of primary structures of various lipoygenases showed that several arginine residues are highly conserved. Some such arginine residues locate in a histidine rich region (Shibata *et al.*, 1988) and the C-ter-

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minimal highly conserved region, and some are reported to locate in two cavities which are postulated as a substrate binding site and an oxygen path (Boyington *et al.*, 1993). Taken together, there is evidence that arginine residues have important role in the enzyme reaction of L-1. Thus, arginine residues of L-1 were modified with an arginine-directed chemical modifier, 2,3-butanedione, and biochemical properties of the modified L-1 were examined.

## Materials and Methods

### Materials

L-1 was purified from soybean seeds (*Glycine max* L. Tamahomare) essentially as described by Axelrod *et al.* (1981). All the experiments described in this paper have been run with a purified enzyme having a specific activity of about 100 U/mg protein. One unit of the enzyme was expressed as the amount of enzyme forming 1  $\mu$ mol of hydroperoxide/min at 25 °C. Linoleic acid (99% pure) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by silica gel column chromatography prior to use. Linoleyl alcohol was prepared by reduction of linoleic acid with lithium aluminum hydride. A chemical modifier, 2,3-butanedione, was a product of Wako Pure Chemical Industries, Osaka, Japan. All other chemicals were purchased from commercial sources.

### Enzyme assay

Activities of L-1 were determined spectrophotometrically at 25 °C by following the formation of linoleic acid hydroperoxide at 234 nm ( $\epsilon = 25,000 \text{ M}^{-1}\text{cm}^{-1}$ ) in 200 mM sodium borate, pH 9.0 or 5 mM sodium borate, pH 8.0 unless otherwise stated. To 2.965 ml of the buffer, 25  $\mu$ l of the substrate solution (10 mM linoleic acid dispersed with 0.2% Tween 20, final concentration; 83.3  $\mu$ M linoleic acid and 0.0017% Tween 20) was added, then the reaction was started with the addition of 10  $\mu$ l of the enzyme solution.

### Modification of lipoxygenase-1 with 2,3-butanedione

Reagents and buffers were prepared fresh daily. Purified L-1 was concentrated by precipitation with ammonium sulfate and redissolved in and

dialyzed against 10 mM sodium phosphate buffer, pH 6.8. Unless otherwise specified, modification was carried out with 0.5 mg/ml of L-1 and 5 mM 2,3-butanedione in 50 mM sodium borate, pH 9.0 at 30 °C. The reaction was initiated by adding 50  $\mu$ l of 10 mM 2,3-butanedione dissolved in 100 mM sodium borate, pH 9.0 to 50  $\mu$ l of the enzyme solution (1 mg/ml, prepared by dilution of 10 mg/ml stock enzyme solution with distilled water). At suitable time intervals, samples were removed and diluted 100-fold with 50 mM sodium borate, pH 9.0 and immediately assayed. For protection of L-1 from modification with 2,3-butanedione, 1 mg/ml lipoxygenase-1 was preincubated with 0.5 mM linoleic acid dispersed in 0.01% Tween 20 for 10 min at 30 °C. Subsequently, an equal volume of 2,3-butanedione solution (10 mM) was added to start the modification reaction. The activity of the modified enzyme was expressed in % to the activity of the intact enzyme that was incubated under the identical conditions without 2,3-butanedione. Product specificities of modified and intact L-1 were analyzed as described previously (Matsui *et al.*, 1992).

### Other methods

Amino acid analysis was carried out with L-1 modified for 20 min with 2,3-butanedione under the standard conditions described above. Modified L-1 was transferred into 30% acetic acid to stop further reaction and to stabilize the modified arginine. After dialysis against 30% acetic acid, the solvent was removed and the residue was hydrolyzed with HCl vapor for 72 hr and the amino acid content was analyzed with an amino acid analyzer (L-8500, Hitachi Co. Japan). Protein content was determined by the modified method of Lowry (Dulley and Grieve, 1975). *cis*-Parinaric acid was used to monitor physical state of linoleic acid in aqueous solution as a hydrophobicity probe (Sklar *et al.*, 1977). Into 3 ml of 5 mM sodium borate, pH 8.0 or 0.2 M sodium borate, pH 9.0 containing various concentration of linoleic acid and Tween 20 (linoleic acid to Tween 20 ratio was kept constant), 5  $\mu$ l of 2.4 mM *cis*-parinaric acid dissolved in ethanol supplemented with 2.4 mM butylated hydroxytoluene was added. Fluorescence derived from *cis*-parinaric acid was monitored with a Hitachi fluorometer (model 056-10S) with excitation

at 325 nm (slit width 3 mm) and emission 420 nm (slit width 6 mm) at 25 °C.

## Results

### Inactivation of L-1 by 2,3-butanedione

Fig. 1 shows the time course of the remaining activity of L-1 after addition of 5 mM 2,3-butanedione to L-1 (0.5 mg/ml) in 50 mM sodium borate, pH 9.0. Although the activity changed little when the assay was performed with 200 mM sodium borate, pH 9.0, with decreasing either the salt concentration or pH value of the assay buffer, a slight inactivation was observed. Decrease in the salt concentration from 200 mM to 5 mM made the inactivation more pronounced than that in pH value from 9.0 to 8.0. When assays were performed with 5 mM sodium borate, pH 8.0, which is a sub-optimal condition for L-1 assay, and only 61.3% of the activity observed with 200 mM, pH 9.0 could be detected with intact L-1, rapid inactivation was evident. With either the longer incubation (up to 180 min) or the higher concentration of 2,3-butanedione (up to 50 mM), about 20% of the original activity still remained. This value seemed to depend on the assay condition, that is, with the

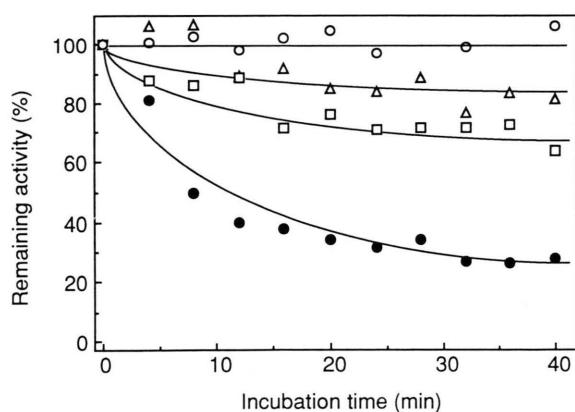


Fig. 1. Time course of inactivation of L-1 by 2,3-butanedione. L-1 (0.5 mg/ml) was reacted with 5 mM 2,3-butanedione in 50 mM sodium borate, pH 9.0 at 30 °C. An aliquot was removed at the indicated time and assayed for the enzyme activity in 200 mM sodium borate, pH 9.0 (○), 200 mM sodium borate, pH 8.0 (△), 5 mM sodium borate, pH 9.0 (□), or 5 mM sodium borate, pH 8.0 (●). Activities of intact L-1 observed under respective assay conditions (115.8, 112.8, 114.4, and 71.0 U/mg for 200 mM/pH 9.0, 5 mM/pH 9.0, 200 mM/pH 8.0, and 5 mM/pH 8.0, respectively) were defined as 100%.

enzyme thoroughly treated with the reagent about 80 or 65% of the original activities were still detected when assays were performed with 200 mM borate buffer, pH 8.0 or 5 mM, pH 9.0, respectively. Amino acid analysis of L-1 modified for 20 min revealed that 6.2 residues of arginine of total 37 (Shibata *et al.*, 1987) were modified and other residues were not. Another chemical modifier directed for an arginine residue, phenylglyoxal, also inactivated this enzyme at 5 mM. Taken together, it was indicated that the inactivation was caused actually by modification of arginine residue(s). As shown in Fig. 2, the rate of inactivation followed pseudo-first order kinetics and was a function of the reagent concentration. The reaction order with respect to 2,3-butanedione was determined as 1.29 from double-log plots of the pseudo first-order-rate constants as a function of reagent concentration (Fig. 2, inset). This kinetic value indicated

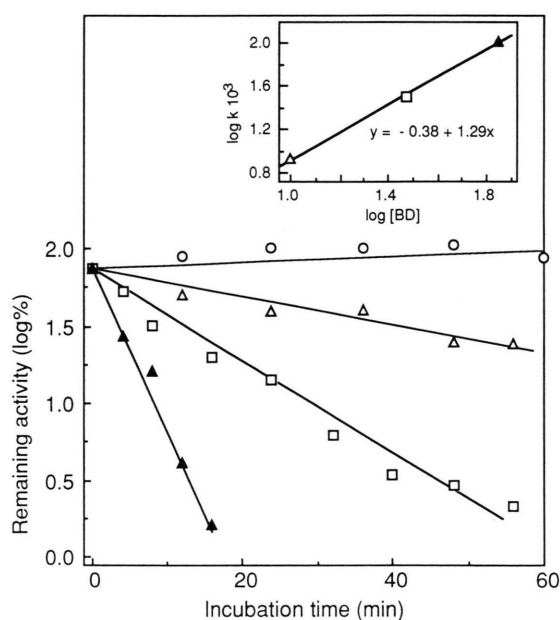


Fig. 2. Time course of inactivation of L-1 by various concentration of 2,3-butanedione. L-1 (0.5 mg/ml) was incubated with 0 (○), 1 (△), 3 (□), or 7 (▲) mM 2,3-butanedione in 50 mM sodium borate, pH 9.0 at 30 °C. An aliquot was removed at the indicated time and assayed for the enzyme activity in 5 mM sodium borate, pH 8.0. Because about 20% of the initial activity persisted even if strict modification condition was adopted, value of 20 was subtracted from those of relative remaining activities (%), then log of the numbers were plotted. *inset*: Plot of log of the pseudo-first-order rate constant (*k*) against log of 2,3-butanedione concentration.

that the inactivation of L-1 by 2,3-butanedione was essentially caused by modification of one arginine residue of L-1.

Riordan (1973) reported that the modification of an arginine residue by 2,3-butanedione resulted in the formation of a borate complex of 4,5-dimethyl-4,5-dihydroxy-2-imidazoline and that this reaction is readily reversible. If the inactivation of L-1 with 2,3-butanedione was caused by the formation of this adduct, the inactivation would be reversed by the removal of borate from the reaction mixture. In order to confirm this possibility, L-1, which had lost about 75% of the original activity as a consequence of exposure to 2,3-butanedione in the presence of borate for 62 min was diluted 100-fold with 50 mM pyrophosphate, pH 9.0 and incubated further at 30 °C. As shown in Fig. 3, the activity of L-1 was restored to its original level within 20 min. This result indicated that the arginine-2,3-butanedione-borate adduct was formed and responsible for the inactivation of L-1, and that an irreversible denaturation of L-1 little occurred during the modification process.

Although the results shown above unequivocally indicated that the modification of arginine residues accounted for the inactivation of L-1, it was thought that the modification occurred at other than around the active center of L-1. Thus,

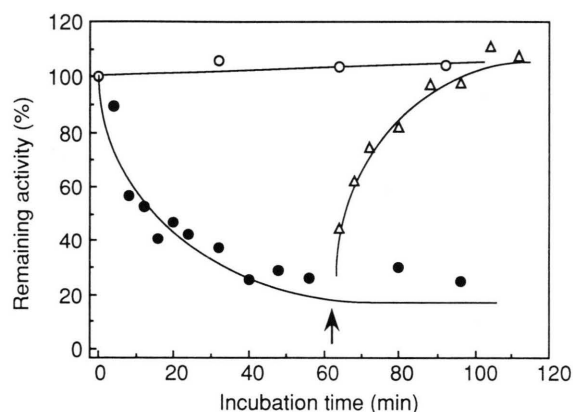


Fig. 3. Reversibility of inactivation of modified lipoxigenase-1. L-1 (0.5 mg/ml) was reacted with (●) and without (○) 5 mM 2,3-butanedione in 50 mM sodium borate, pH 9.0. After 62 min of modification, aliquots were diluted 100-fold with 50 mM sodium pyrophosphate, pH 9.0 and incubated further at 30 °C (△). L-1 activities were assayed with 5 mM sodium borate, pH 8.0 at the indicated time.

we tried to protect the modification by concomitant incubation with a substrate, linoleic acid, during modification reaction. As shown in Fig. 4, linoleic acid effectively protected this enzyme from the inactivation by 2,3-butanedione. A substrate analog, linoleyl alcohol which showed reactivity of only about 10% of that of linoleic acid under the standard assay condition, exerted only a little protective effect. The other substrate analogs such as undecylenic acid or oleic acid, which are not substrates for L-1, did not protect L-1 even if these analogs were used at 2.5 mM. These results suggested that the protective effect caused by linoleic acid could be accounted for a specific substrate-enzyme recognition of L-1. Thus, modified arginine residue(s) responsible to the inactivation is suggested to locate around the active center of L-1.

#### *Properties of modified L-1*

(1) pH-activity profile. As shown in Fig. 5, intact L-1 showed broad pH-activity curve. After modification, the curve became narrower, and lower activity was evident at lower pH. That is, at pH 9.5 or higher modification caused only about 5–20% inactivation, while the rate of inactivation increased with decreasing pH value of the assay mixture. At pH 8.0 about 70% of the initial activity was lost.

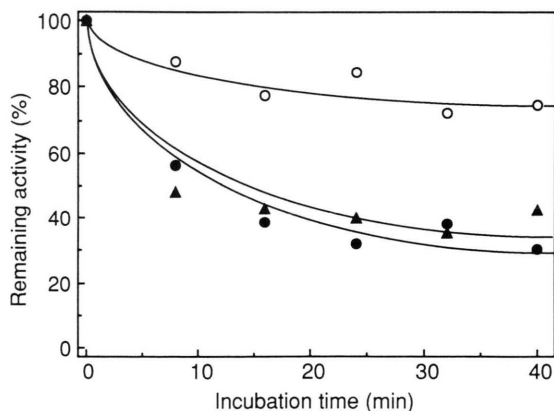


Fig. 4. Protection of L-1 from inactivation. L-1 (1 mg/ml) was pre-incubated with 0.5 mM linoleic acid (○), 0.5 mM linoleyl alcohol (▲), or without protecting reagent (●) for 10 min at 30 °C, and modification reaction was started with the addition of equal volume of 10 mM 2,3-butanedione in 100 mM sodium borate, pH 9.0. L-1 activity was assayed with 5 mM sodium borate, pH 8.0.



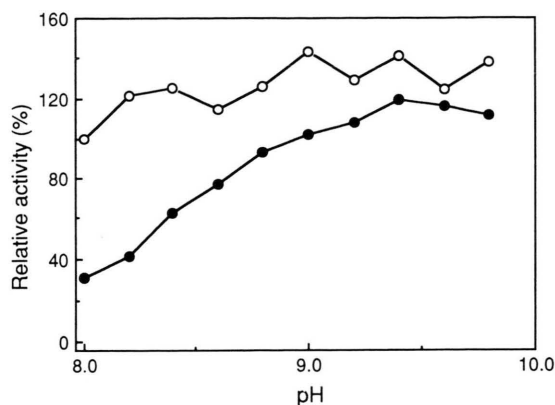


Fig. 5. pH-activity curves of intact and modified L-1. L-1 (0.5 mg/ml) was reacted with (●) and without (○) 5 mM 2,3-butanedione in 50 mM sodium borate, pH 9.0 for 60 min. The reaction was stopped with diluting the reaction mixture 100-fold with 50 mM sodium borate, pH 9.0. L-1 activity was assayed with 20 mM sodium borate of respective pH. 9.0.

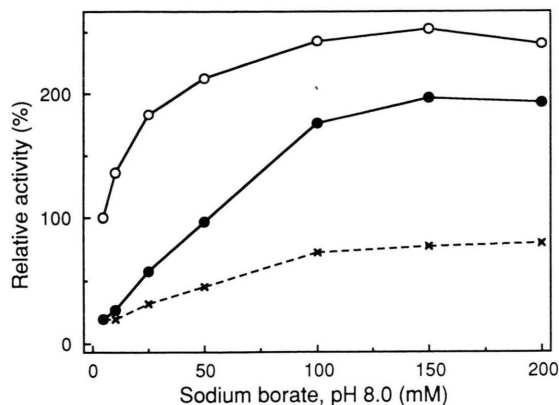


Fig. 6. Effect of salt concentrations on intact and modified L-1. L-1 (0.5 mg/ml) was reacted with (●) and without (○) 5 mM 2,3-butanedione in 50 mM sodium borate, pH 9.0 for 40 min. The activities were determined with the indicated concentration of sodium borate, pH 8.0. The activity of intact L-1 with 5 mM sodium borate, pH 8.0 (45.6 U/mg) was defined as 100%. Relative remaining activities (×) were also plotted with dotted lines.

(2) Effect of salt concentration. Effect of arginine modification of L-1 was most pronouncedly observed when dependence of the activity on salt concentration was assayed. Increase of sodium borate concentration enhanced the activity of both intact and modified L-1 but differently. With modified L-1 the activity increased linearly up to 100 mM where about 10-fold of the activity was observed. On the other hand, with intact L-1 hyperbolic curve was obtained, and the enhancement was only two-fold (Fig. 6). This is not specific only to sodium borate. With addition of KCl to 5 mM sodium borate, both the activity increased. Again, the enhancement saturated lower concentration with intact L-1 than with modified one. Furthermore, while the activity of intact L-1 increased only two-fold, that of modified L-1 did five-fold.

(3) Product specificity. Because it was expected that the modified arginine residues of L-1 bound the terminal carboxylate anion of a substrate during its catalysis as proposed by Gardner (1989), product specificities of modified and intact L-1 were compared. The reaction was performed with linoleic acid in 5 mM sodium borate, pH 8.0 in order to differentiate each other more effectively. Intact L-1 showed high product specificity, i.e., 13-hydroperoxy-(9Z,11E)-/13-hydroperoxy-(9E,11E)-/9-hydroperoxy-(10E,12Z)-/9-hydroper-

oxy-(10E,12E)-octadecadienoic acid was 94.8/1.3/2.7/1.3. This value is equivalent to the value observed with 0.2 M sodium borate, pH 9.0 (Matsui *et al.*, 1992) although Gardner (1989) observed a slight increase of 9-hydroperoxide ratio at pH 8.0. Unexpectedly, the modified L-1 showed almost the same product specificity, i.e., 13-hydroperoxy-(9Z,11E)-/13-hydroperoxy-(9E,11E)-/9-hydroperoxy-(10E,12Z)-/9-hydroperoxy-(10E,12E)-octadecadienoic acid was 95.0/1.0/3.0/1.0. Thus, it was indicated that orientation of a substrate in the reaction center of L-1 is not affected by the modification of arginine residue(s) although the activity under the assay conditions highly differed.

(3) S-V plots. Both the intact and modified L-1 showed S-V plot almost obeying Michaelis-Menten kinetics when assays were performed in 0.2 M sodium borate, pH 9.0. Double reciprocal plots revealed that  $k_m$  values were 29.2 and 26.7  $\mu\text{M}$  for intact and modified L-1, respectively (not shown). When assays were performed in 5 mM sodium borate, pH 8.0, S-V plot of both the intact and modified L-1 were not hyperbolic. Double-reciprocal plot for the intact L-1 showed a symptom of substrate inhibition. On the other hand, the activity of modified L-1 proportionally increased as the concentration of linoleic acid increased at least within the concentration exam-

ined. It should be noticed that with higher concentration range, where activity of intact L-1 increased only in a lesser extent, the activity of modified L-1 increased still linearly.

#### Physical state of linoleic acid

Because the substrate, linoleic acid, is amphiphilic, increase of the concentration would cause formation of aggregates such as acid-soap and micelle (Verhagen *et al.*, 1978). Formation of such aggregates has been reported to affect L-1 activity (Verhagen *et al.*, 1978; Gibian and Colanduoni, 1984). In the case of the modified L-1, changes of the physical state of linoleic acid was thought to be one of the reasons why the inactivation was manifested with the specific assay conditions. Thus, the physical state of the substrate in the assay conditions was monitored with *cis*-parinaric acid as a fluorescent, hydrophobic probe (Sklar *et al.*, 1977). *cis*-Parinaric acid is thought to an ideal fluorescent probe because it has structural similarity with linoleic acid. The fluorescence titration curve was rather complicated as shown in Fig. 7. There are at least two points where physical

state of linoleic acid was thought to change both in 200 mM sodium borate, pH 9.0 and 5 mM sodium borate, pH 8.0. The first ones were observed at about 25  $\mu\text{M}$  with both the buffer. When compared with the value determined by surface-tension measurements (Verhagen *et al.*, 1978), they were thought to reflect formation of acid-soap from monomer. The second ones at about 100 and 190  $\mu\text{M}$ , with 200 mM, pH 9.0 and 5 mM pH 8.0, respectively, were thought to reflect formation of micellar aggregate from acid-soap although the values are smaller than those reported with surface-tension measurement (Verhagen *et al.*, 1978). Because final concentration of linoleic acid was 83.3  $\mu\text{M}$ , these analyses indicated that in both the assay conditions acid-soap form was most abundant species, and that the acid-soap to monomer ratio were not so different each other. Thus, observed difference of the modified L-1 activity depending on the assay buffer seemingly did not derived from difference in the physical state of the substrate.

#### Discussion

Although amino acid analysis indicated that arginine residues of L-1 were readily modified with 5 mM 2,3-butanedione, effect of modification was only observed with sub-optimal assay condition of L-1, i.e., 5 mM sodium borate, pH 8.0. Thus, significance of the modified arginine residues for enzymatic catalysis was seemed not so great. Gardner (1989) suggested that there should exist a site recognizing carboxylate anion of a substrate fatty acid in L-1. In many enzymes an arginine residue is known to bind a carboxylate anion of a substrate in its substrate-recognition site. Thus, an arginine residue was seemed to be most probable candidate for the binding site in L-1. Nonetheless, comparison of product specificities of intact and modified L-1 unambiguously indicated that arginine residue(s) do not participate in recognition of the carboxylate anion to locate a substrate in a proper orientation in the substrate binding site of L-1.

Because of its amphiphilic nature, linoleic acid would aggregate to form acid-soap and micelle in aquatic solution depending on its concentration. Some kinds of enzymes which act on amphiphilic substances, for example, phospholipase  $A_2$ , are

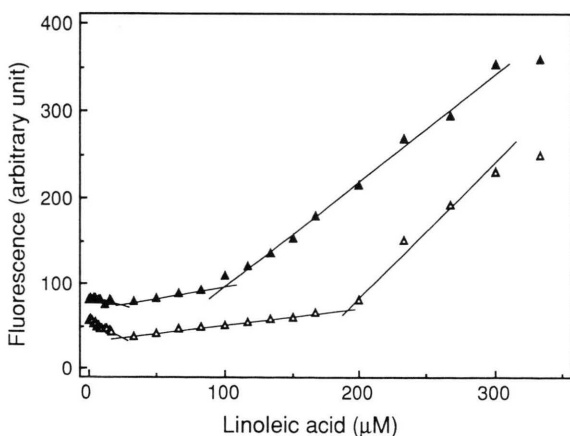


Fig. 7. Fluorimetric titration of *cis*-PA with linoleic acid. Into 3 ml of 5 mM sodium borate, pH 8.0 ( $\Delta$ ) or 0.2 M sodium borate, pH 9.0 ( $\bullet$ ) containing various concentration of linoleic acid and Tween 20 (linoleic acid to Tween 20 ratio was kept constant and was the same as that of standard L-1 activity assay condition), 5  $\mu\text{l}$  of 2.4 mM *cis*-parinaric acid dissolved in ethanol supplemented with 2.4 mM butylated hydroxytoluene was added. Fluorescence derived from *cis*-parinaric acid was monitored with a Hitachi fluorometer with excitation at 325 nm (slit width 3 mm) and emission at 420 nm (slit width 6 mm) at 25  $^{\circ}\text{C}$ .

known to act preferentially on a substrate aggregates such as micelles, vesicles (Scott *et al.*, 1990). Because inactivation of L-1 upon modification was observed only with a buffer of low salt concentration and low pH, changes in physical state of the substrate were a next candidate which made inactivation visible. Fluorimetric titration of linoleic acid by using *cis*-parinaric acid showed that with the concentration of linoleic acid used for the assay (83.3  $\mu$ M) about 2/3 of the substrate forms acid-soap aggregate and 1/3 exists as a monomer in both the assay mixtures. Thus, this hypothesis should also be discarded.

It is well known that enzyme activities are frequently affected by the salt strength of the assay solution. This is attributable to changes of activity coefficients of ions depending on Debye-Huckel's theory. In this study, it was shown that modification of arginine residue(s) changed response of L-1 against salt concentration and pH. These factors must affect electrostatic interaction within L-1 and between L-1 and a substrate. Thus, modi-

fied arginine residue(s) was thought to have a role in supporting ideal electrostatic interaction even in sub-optimal reaction conditions. Although arginine residues are not involved directly in binding nonheme iron in L-1 (Boyington *et al.*, 1993), three arginine residues (Arg515, 532 and 541) locate in and around the histidine rich region and are highly conserved within lipoxigenases of various origins. Furthermore, Arg707 locates in the middle of the cavity II which is postulated to be a substrate pocket, and Arg580 locates in the cavity I which is postulated to be a molecular oxygen path (Boyington *et al.*, 1993). These are also highly conserved in lipoxigenases. In order to certify the role of the arginine residues quantitatively, site-directed mutagenesis should be performed.

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