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Interaction of *Rhizopus delemar* Lipase with 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane and Structurally Related Pesticides: Importance of 1:1 Pesticide–Lipase Complexes[1)]

HIROYUKI KANEKI, MIZUO NAKAUCHI and MITSUYA TANAKA*

School of Pharmaceutical Science, Toho University, Miyama 2-2-1, Funabashi 274, Japan

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The interaction of *Rhizopus delemar* C-lipase with 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane (DDT) and structurally related pesticides was studied. Binding assays and titration experiments revealed that the binding is distinctly cooperative and the 1:1 pesticide–lipase complex formation governs the ensuing complex formation. The chlorinated pesticides used here fall into three groups according to the mode of interaction with lipase. (1) DDT, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE), the 1:1 pesticide–lipase complexes of which bind 8 further molecules of any pesticide used here to form 9:1 complexes with much higher activity. (2) Dimic and dichlorobenzophenone, the 1:1 pesticide–lipase complexes of which can bind 8 further molecules of the same pesticide without activation, but cannot bind any other pesticide. (3) Kelthane, chlorobenzilate and chloropropylate, which are non-competitive inhibitors of the intact lipase but behave as activators for 1:1 pesticide–lipase complexes of pesticides in group (1).

The formation of the very tightly bound 9:1 DDT–lipase complex at pH 6.0 was found to be pH-dependent, and the bound pesticide was completely recovered at pH 4.5. Chromatography on CM-Sephadex C25 gave results supporting the one-step formation of a 9:1 DDT–lipase complex from a 2:1 DDT–lipase complex.

Keywords——*Rhizopus delemar*; lipase; pesticide; DDT; complex formation; stoichiometry; cooperativity; activation; inhibition

The interaction between phospholipid and enzymes has attracted much attention with regard to the enzyme function, especially of membrane-bound enzymes.[2)] Lipophilic compounds can be expected to affect the enzyme function, playing a role in the place of phospholipid, and the interaction of enzymes with lipophilic xenobiotics may have interesting consequences.

Rhizopus delemar C-lipase (*Rh.* lipase) is known to bind phosphatidylcholine to form a 5:1 phosphatidylcholine–lipase complex having much higher activity than the intact enzyme toward lipoprotein and water-soluble artificial esters.[3)] *Rh.* lipase is unique among lipases from various sources in that some chlorinated pesticides bind to the lipase to form stoichiometrically and tightly bound pesticide–lipase complexes with highly elevated activity toward tripropionin.[4)] This activation is not observed with triglycerides or esters of long-chain fatty acid. A stepwise mechanism for the pesticide–lipase complex formation was presented for 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) and aldrin.[4)] As shown in Chart 1

$$\mathrm{E} \xrightarrow[\text{fast}]{} \mathrm{E'\text{-}DDT_2} \xrightarrow[\text{slow}]{} \mathrm{E\text{-}DDT_2} \xrightarrow[\text{very fast}]{} \mathrm{E^*\text{-}DDT_9}$$

Chart 1

for DDT, two molecules of DDT first bind to a molecule of lipase (E) to form a precursor 2 : 1 DDT–lipase complex (E′-DDT_2), which is slowly transformed into a 2 : 1 complex (E-DDT_2), and seven further molecules of DDT bind simultaneously to E-DDT_2 to afford a 9 : 1 DDT–lipase complex (E*-DDT_9). Only E*-DDT_9 has an elevated activity toward tripropionin (pesticide–lipase complexes with elevated activity are asterisked in this paper).

This paper demonstrates the importance of 1 : 1 pesticide–lipase complexes that govern the ensuing complex formation and activation of the lipase.

Materials and Methods

Preparation of Enzyme——Purification of *Rh.* C-lipase was performed according to the method of Iwai and Tsujisaka[5] except that the starting material was a commercial product of *Rh.* lipase (Seikagaku Kogyo Co., Ltd.). The homogeneity of the purified enzyme was confirmed by disc electrophoresis on 7.5% polyacrylamide gel (pH 4.0). The concentration of the lipase was determined spectrophotometrically, based on a molar extinction coefficient of 5.09×10^4 at 280 nm.[4] The molecular weight was 45000 as estimated by gel filtration, in good agreement with the value reported by Shimada *et al.*[3b]

Reagents——Commercial tripropionin (Tokyo Kasei Kogyo Co., Ltd.) was purified by passage through an Al_2O_3 column by the method of Entressangles and Desnuelle.[6] Chloroethane pesticides and structurally related pesticides, DDT, 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (DDE), 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol (kelthane), ethyl 4,4'-dichlorobenzilate (chlorobenzilate), isopropyl 4,4'-dichlorobenzilate (chloropropylate), 2,2-bis(4-chlorophenyl)ethane (dimic) and 4,4'-dichlorobenzophenone (dichlorobenzophenone) were purchased from Wako Pure Chemical Industries Co., Ltd. CM-Sephadex C25 was from Pharmacia Fine Chemical Co., Ltd. All the other materials used here were special-grade reagents purchased from Nakarai Chemicals Co., Ltd.

Assay of Lipase Activity——Lipase activity was assayed as described previously.[4] The rate of hydrolysis of tripropionin (a water-soluble substrate) was determined by measurement of free acid liberated in an unbuffered reaction mixture (10 ml) containing 1 nmol of lipase and 2.93 mM tripropionin at pH 6.0 at 30 °C in a CO_2-free air stream. The volume of 0.01 N NaOH used to neutralize liberated acid was recorded as a function of time with a pH-stat(model PS-11, Hiranuma Sangyo Co., Ltd.). The titration values were corrected with regard to the fact that 91% of liberated propionic acid was titrated at pH 6.0. Initial velocity, v, was determined from the linear time course at less than 5% hydrolysis, the consumption of titrant being less than 140 μl.

Binding Assays by Isopropyl Ether Treatment——Extraction of unbound (or weakly bound) pesticide from an incubation mixture of lipase and pesticide was carried out as follows. An EtOH solution (20 μl) containing 10—200 nmol of pesticide was added to an unbuffered aqueous solution (50 ml) containing 0.45 mg (10 nmol) of lipase, and then this mixture was incubated. Unless otherwise stated, incubation of lipase with pesticide was carried out at pH 6.0 at 30 °C for 6 s. The incubation mixture was extracted three times by shaking for 10 min with 50 ml each of isopropyl ether as described previously.[4]

For the determination of unbound pesticide in the mixture, the isopropyl ether layer was evaporated to dryness. The residue was dissolved in a small amount of isopropyl ether. The kinds of pesticides were identified from ultraviolet spectra at 220—300 nm, and the amounts of pesticide were determined by the measurement of absorbance at 240 nm.[7a] These spectra were similar to those measured in hexane.[7b]

For the measurement of lipase activity in the water layer, the water layer was kept under reduced pressure at 15 °C for 15 min to remove the remaining solvent and the lipase activity was assayed at pH 6.0 at 30°C. Treatments with isopropyl ether had no adverse effect on the lipase activity.

Titration Experiments——Various amounts of pesticide were added to a fixed amount of lipase, and the mixture was preincubated for 6 s. The mixture was assayed for lipase activity directly or after removal of excess pesticide by the isopropyl ether treatment described above. Titration curves were obtained by plotting the relative activity against the amount of pesticide.

Effect of pH on Pesticide–Lipase Complexes——An unbuffered solution (50 ml) containing 10 nmol of lipase was incubated with 20 or 90 nmol of pesticide at pH 6.0 for 6 s. The incubation mixture (a solution of 2 : 1 or 9 : 1 pesticide–lipase complex) was adjusted to a desired pH with 0.05 N HCl or NaOH. After standing for 10 min, unbound pesticide was determined by isopropyl ether treatment as described above. Lipase activity in the water layer was measured after removal of the remaining solvent and adjustment of the pH to 6.0.

Column Chromatography on CM-Sephadex C25——After isopropyl ether treatment of a mixture of lipase and pesticide, the water layer was concentrated by ultrafiltration and used as a solution of pesticide–lipase complex. Intact lipase or pesticide–lipase complex (each 2.25 mg protein) was applied to a CM-Sephadex C25 column (1 × 15 cm) equilibrated with 0.01 M acetate buffer (pH 6.0) at 30 °C. Elution was carried out with a linear gradient increase of NaCl concentration and fractions of 2 ml were collected at a flow rate of 2 ml per hour.

Results

Effect of Substrate Concentration

The effects of substrate concentrations from 0.56 to 3.18 mM on the enzymatic hydrolysis rates of tripropionin in the presence of pesticide were studied. Values of Michaelis constant (K_m) and maximum velocity (V_{max}) calculated from Lineweaver–Burk plots are summarized in Table I.

Chloroethane pesticides and structurally related pesticides examined here could be divided into three groups according to the effect on the lipase activity. (1) DDT, DDD and DDE, having chlorine atoms at the position 1 in addition to the chlorine atoms on the benzene rings, enhanced the lipase activity, increasing the V_{max} value while the K_m value remained unchanged. (2) Dimic and dichlorobenzophenone, having chlorine atoms only on the benzene rings, had no effect on the lipase activity. (3) Kelthane, chlorobenzilate and chloropropylate, having a hydroxy group at position 1, exhibited non-competitive inhibition as judged from Lineweaver–Burk plots and Dixon plots. Chlorobenzilate and chloropro-

TABLE I. Effects of Chloroethanes and Related Compounds on the Lipase-Catalyzed Hydrolysis of Tripropionin[a)]

Added pesticide (10 μM)	Michaelis constant[a)] (mM)	Maximum velocity[a)] (μmol free acid/min/mg protein)	Effect on lipase activity (complex formed)
None	3.8±0.18	20±0.4	
DDT[b)]	3.8±0.15	90±2.4	4.4-Fold activation
DDD[b)]	3.8±0.23	89±1.8	(9:1 pesticide–lipase complex)
DDE[b)]	3.8±0.10	91±2.0	
Dimic[b)]	3.8±0.20	20±0.2	No effect
Dichlorobenzophenone	3.9±0.18	20±0.5	(9:1 pesticide–lipase complex)
Kelthane[b)]	3.9±0.11	15±1.1	
Chlorobenzilate	3.9±0.13	13±0.1	Non-competitive inhibition[c)]
Chloropropylate	3.9±0.12	10±0.4	

a) Michaelis constants (K_m) and maximum velocities (V_{max}) with standard deviations were calculated from five to eight Lineweaver–Burk plots, each of which involved seven points. *b*) K_m and V_{max} values are in good agreement with the values in the previous report.[8)] *c*) The inhibition constants (K_i) of kelthane, chlorobenzilate and chloropropylate calculated from Lineweaver–Burk plots and Dixon plots were 30, 19 and 10 μM, respectively.

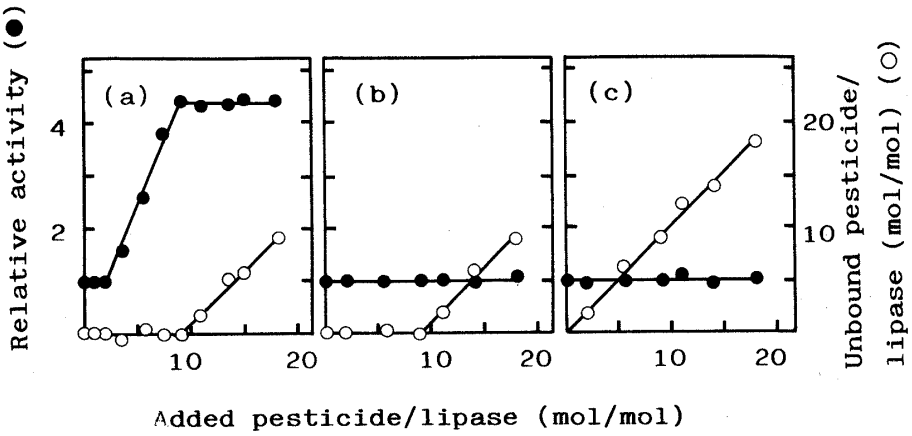

Fig. 1. Binding Assays and Titration Experiments

(a) DDT. (b) Dimic. (c) Kelthane.
●, relative activity; ○, molar ratio of unbound pesticide to lipase.

pylate, although they are esters, cannot be hydrolyzed by the lipase.

The same classification described above was valid throughout the following experiments. Therefore, only the data obtained with DDT, dimic and kelthane are shown.

Binding Ability and Effect on Lipase Activity of Pesticides

The binding ability of pesticides to lipase and the effect on lipase activity were examined by means of binding assays and titration experiments as follows. A preincubation mixture of lipase and pesticide was extracted with isopropyl ether to remove the unbound (or weakly bound) pesticide, and then the amount of pesticide extracted with isopropyl ether and the lipase activity in the water layer were determined. The results for DDT, dimic and kelthane are shown in Fig. 1.

As shown in Fig. 1a, unbound DDT was extracted with isopropyl ether only at more than 9 : 1 molar ratios of DDT to lipase, indicating the absence of free DDT in the preincubation mixture, that is, complete binding of pesticide added up to a 9 : 1 molar ratio. The lipase activity in the water layer increased linearly with the amount of added DDT up to the 9 : 1 molar ratio after a "plateau" (a horizontal portion of the titration curves) up to a 2 : 1 molar ratio. The same titration curve was reported previously where the lipase activities were assayed without removal of unbound DDT by isopropyl ether treatment.[4] With DDD and DDE, the same results were obtained.

As shown in Fig. 1b, the unbound dimic was also extracted with isopropyl ether only at more than 9 : 1 molar ratios of dimic to lipase while the lipase activity in the water layer did not change with or without isopropyl ether treatment. With dichlorobenzophenone, the same result was obtained. This indicates the tight binding of dimic or dichlorobenzophenone up to a 9 : 1 molar ratio of pesticide to lipase without activation of the lipase activity, in contrast to the case of DDT.

Experiments with kelthane revealed a different situation. As shown in Fig. 1c, all the added kelthane was extracted with isopropyl ether and the lipase activity in the water layer did not change. With chlorobenzilate and chloropropylate, the same results were obtained. This indicates the weak reversible binding of these pesticides to lipase, as expected from the K_i values of these pesticide, 10 to 30 μM (note (c) in Table I).

The binding of pesticide to lipase was found to be independent of incubation time when the time of incubation of lipase with pesticide was varied in the range of 0.1 to 60 min. Incubation at least for 6 s was necessary to obtain reproducible results, however.

Evidence of 1 : 1 Pesticide–Lipase Complexes

We had previously suggested that E′-DDT_2 changes very slowly to E-DDT_2 and that the resultant E-DDT_2 binds further DDT molecules much more rapidly than intact lipase to form the activated complex, E*-DDT_9, exclusively (Chart 1).[4] If this is the case, complex behavior of the lipase towards DDT will be expected when DDT is added to the lipase portionwise with an interval that allows the formation of E-DDT_2 from E′-DDT_2.

No enhancement of lipase activity occurs when 2 nmol of DDT is added at once to 1 nmol of lipase (formation of E-DDT_2). Curiously, however, a 1.29-fold increase in activity was observed when 0.5 nmol of DDT was added to a preincubation mixture (6 s) of 1.25 nmol of DDT and 1 nmol of lipase (therefore, 1.75 nmol of DDT being added in total) (Fig. 2). This portionwise addition of DDT provided evidence for the formation of E-DDT prior to the formation of E-DDT_2.

Lipase (1 nmol) was preincubated with DDT (0.5—1.75 nmol) for 6 s, and then various amounts of DDT were added. After incubation for 6 s, the enzyme reaction was initiated by addition of substrate.

As shown in Fig. 2, with preincubation mixtures of DDT and lipase at the molar ratios of 0, 0.5 and 1.0, the lipase activity increased only after the plateau corresponding to the addition

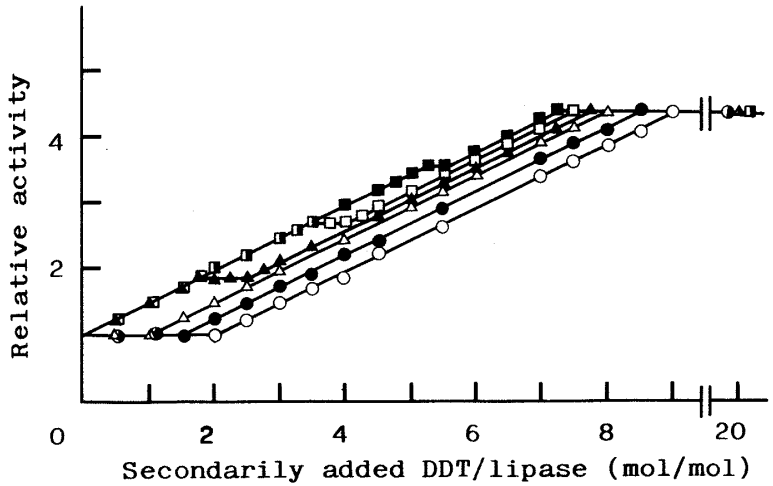

Fig. 2. Effect of DDT on the Activity of a Preincubation Mixture of Lipase and Various Amounts of DDT

Amounts of DDT firstly added to lipase (1 nmol): ○, 0 nmol; ●, 0.50 nmol; △, 1.00 nmol; ▲, 1.25 nmol; □, 1.50 nmol; ■, 1.75 nmol.

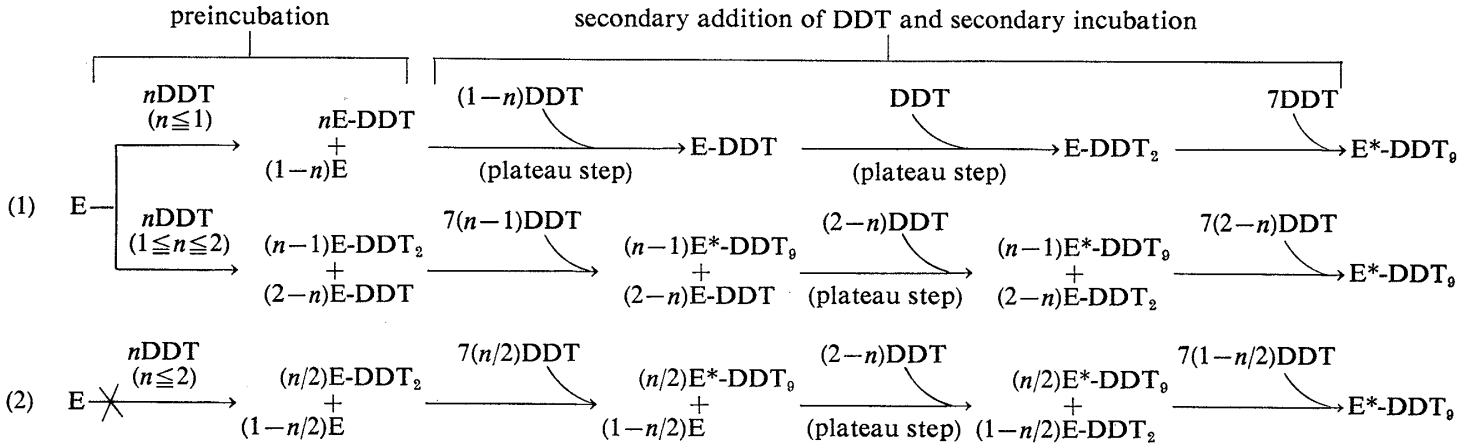

Chart 2

A step in which the amount of E*-DDT$_9$ remains constant corresponds to a "plateau" in activation.

of 2.0, 1.5 and 1.0 nmol of DDT, and attained the maximum activation level of E*-DDT_9 on addition of a further 7 nmol of DDT. However, with preincubation mixtures of DDT and lipase at the ratio of 1.25, 1.50 and 1.75, the lipase activity increased promptly with increasing amounts of secondarily added DDT, and a plateau in activation appeared later at the molar ratios of 1.75—2.50, 3.50—4.00 and 5.25—5.50, respectively. These values agree with those calculated assuming the scheme in route (1) in Chart 2. This indicates that the formation of E-DDT exclusively precedes the formation of E-DDT_2. Alternatively, if two molecules of DDT bind simultaneously to a molecule of lipase to form E-DDT_2 without the formation of E-DDT (route (2) in Chart 2), then the plateaus in activation should occur at the molar ratios of 1.75—3.25, 3.5—4.5, 4.375—5.125, 5.25—5.75 and 6.125—6.375 as calculated from the scheme in route (2) in Chart 2, when DDT was added to preincubation mixtures of DDT and lipase at the molar ratios of 0.50, 1.00, 1.25, 1.50 and 1.75, respectively. With DDD and DDE, the same results were obtained.

Formation of a 2 : 1 pesticide–lipase complex had been thought to be the key step in the process of activation. In the light of the 1 : 1 complex formation described above, the following experiments were carried out with 2 : 1 and 1 : 1 complexes.

Cooperation of Pesticides and Importance of 1 : 1 Pesticide–Lipase Complexes

Dimic seems to bind to lipase much faster than DDT. No activation occurred when a mixture of dimic and DDT was added to lipase solution.

Effect of Dimic on 1 : 1 and 2 : 1 DDT–Lipase Complexes——Various amounts of dimic (0—120 nmol) were added to a solution in which lipase (10 nmol) had been preincubated with

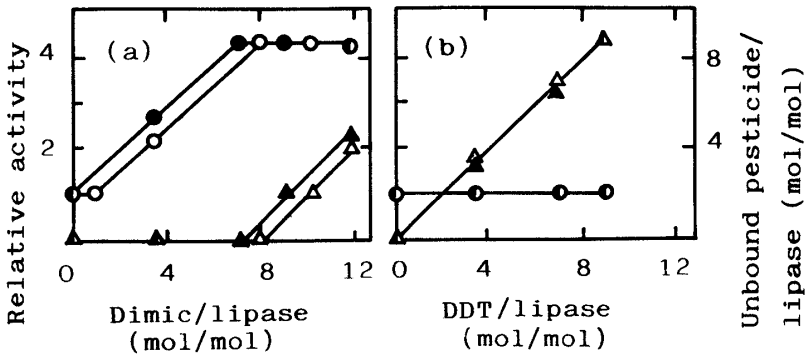

Fig. 3. Cooperation of DDT and Dimic

(a) Various amounts of dimic were added to a mixture of DDT and lipase at a molar ratio 1 : 1 (○, relative activity; △, unbound dimic) or 2 : 1 (●, relative activity; ▲, unbound dimic).

(b) Various amounts of DDT were added to a mixture of dimic and lipase at a molar ratio 1 : 1 (○, relative activity; △, unbound DDT) or 2 : 1 (●, relative activity; ▲, unbound DDT).

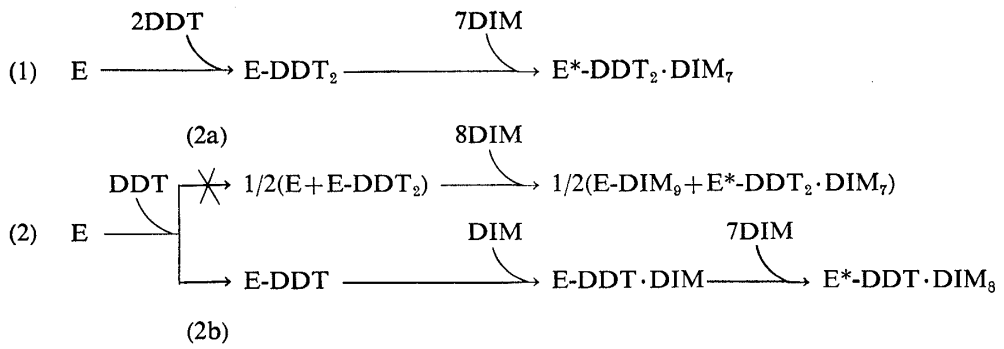

Chart 3

DDT (10 or 20 nmol). After incubation, this mixture was treated with isopropyl ether and then the lipase activity in the water layer was assayed.

As shown in Fig. 3a, when dimic was added to a preincubation mixture of DDT and lipase at a 2:1 molar ratio (E-DDT_2), unbound dimic was extracted with isopropyl ether at more than 7:1 molar ratios of dimic to lipase. The lipase activity in the water layer increased with increasing amount of added dimic and attained a constant value, a 4.4-fold increase of activity, at a 7:1 molar ratio of dimic to lipase. Without isopropyl ether treatment, similar results were obtained. This indicates that seven molecules of dimic bind simultaneously to E-DDT_2 in the same manner as DDT to form a 2:7:1 DDT–dimic–lipase complex (E*-$DDT_2 \cdot DIM_7$) having the same activity as E*-DDT_9, as shown in route (1) in Chart 3.

On the other hand, when dimic was added to the preincubation mixture of lipase and DDT in an equimolar ratio, unbound dimic was extracted at more than 8:1 molar ratios of dimic to lipase. After a plateau up to a 1:1 molar ratio, the lipase activity in the water layer increased linearly with increasing amount of added dimic up to the 8:1 molar ratio, where 4.4-fold activation was observed. At the plateau, a 1:1:1 DDT–dimic–lipase complex (E-DDT·DIM) without elevated activity should be formed from E-DDT (route (2b) in Chart 3). Without isopropyl ether treatment, similar results were obtained.

This again indicates that E-DDT exists in the process of the formation of E-DDT_2 from E, since if E changes directly into E-DDT_2 as shown in route (2a) in Chart 3, the magnitude of activation on addition of dimic should be half as much as the experimental value owing to the formation of a 9:1 dimic–lipase complex (E-DIM_9) and E*-$DDT_2 \cdot DIM_7$ in an equimolar ratio. This consideration also explains the exclusive formation of an activated complex, a 1:8:1 DDT–dimic–lipase complex (E*-DDT·DIM_8) having the same activity as E*-DDT_9 (route (2b) in Chart 3).

When the other pesticides used here, including kelthane, chlorobenzilate and chloropropylate which are non-competitive inhibitors, were used instead of dimic, similar tight binding and 4.4-fold activation were observed.

Effect of DDT on 1:1 and 2:1 Dimic–Lipase Complexes——Various amounts of DDT (0—120 nmol) were added to a solution in which lipase (10 nmol) had been preincubated with dimic (10 or 20 nmol).

As shown in Fig. 3b, all the added DDT was extracted with isopropyl ether, indicating that dimic–lipase complexes cannot bind any DDT molecule (Chart 4). The lipase in the water layer had the same activity as the intact lipase. Without isopropyl ether treatment, similar results were obtained. This result suggests that as shown in route (2a) in Chart 4, a 1:1 dimic-lipase complex (E-DIM) is exclusively formed before the formation of a 2:1 dimic–lipase complex (E-DIM_2) from E, since as shown in route (2b) in Chart 4, addition of DDT should result in activation with the formation of E*-DDT_9 from one-half of the intact lipase if E is transformed directly to E-DIM_2. With all the other pesticides used here, instead of DDT

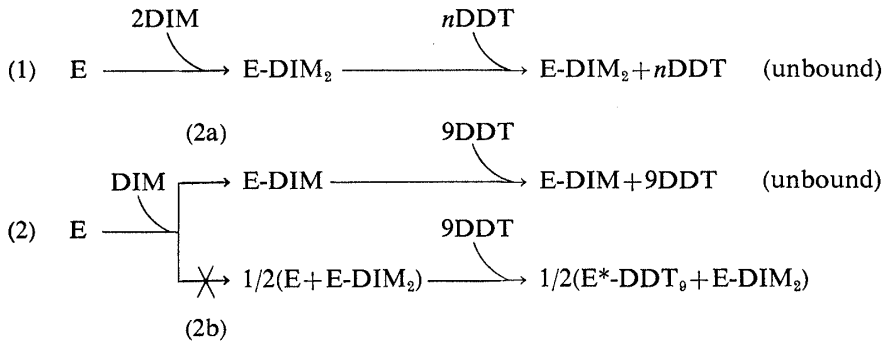

Chart 4

(other than dimic itself), very similar results were obtained. Even dichlorobenzophenone which belongs to the same group as dimic could not bind to E-DIM or E-DIM_2. A similar result was obtained with dichlorobezophenone instead of dimic, and 1:1 and 2:1 dichlorobenzophenone–lipase complexes could bind only dichlorobenzophenone.

Effect of pH on 9:1 Pesticide–Lipase Complexes

The effect of pH on pesticide–lipase complexes was examined as follows. A preincubation mixture of pesticide and lipase (9:1), was adjusted to a desired pH (pH 4.0—7.0) and this solution was treated with isopropyl ether to remove the unbound pesticide.

As shown in the case of DDT in Fig. 4a, release of DDT was observed at pH below 5.0 and was complete at pH 4.5. The lipase activity (assayed at pH 6.0) in the water layer decreased on isopropyl ether treatment at pH below 5.0 and was equal to the activity of intact lipase at pH 4.5. With DDD and DDE, the same results were obtained. As shown in Fig. 4b, similar release of dimic from E-DIM_9 was observed, while the lipase activity (assayed at pH 6.0) in the water layer was equal to the activity of an intact lipase at any pH. With dichlorobenzophenone, the same result was obtained. As shown in Fig. 4c, kelthane, a non-competitive inhibitor, was recovered with isopropyl ether treatment at any pH and the lipase activity in the water layer did not change. With chlorobenzilate and chloropropylate, the same results were obtained.

The lipase in the water layer from which pesticides had been removed completely by isopropyl ether treatment at pH 4.0 behaved in the same way as the intact lipase toward DDT, dimic and kelthane, indicating pH-dependent reversible binding of DDT, DDD, DDE, dimic or dichlorobenzophenone to lipase.

Similarly, pesticides were quantitatively recovered from 2:1 pesticide–lipase complexes.

Column Chromatography on CM-Sephadex C25

As shown in the case of DDT in Fig. 5a, lipase protein appeared as two separate peaks with almost the same size when a mixture of DDT and lipase at the molar ratio of 5.5 was applied to a column of CM-Sephadex C25. The mixture is expected to give E-DDT_2 and E*-DDT_9 in an equimolar ratio when only the formation of E*-DDT_9 and E-DDT_2 is assumed. The first peak had 4.4 times the specific activity of the intact lipase and the second peak had the same specific activity as the intact lipase. The molar ratios of DDT to lipase were determined to be 9:1 for the first peak and 2:1 for the second peak by isopropyl ether treatment at pH 4.0 as described above. A solution of E*-DDT_9 and a solution of E-DDT_2 exhibited essentially the same elution volume as the first peak and the second peak,

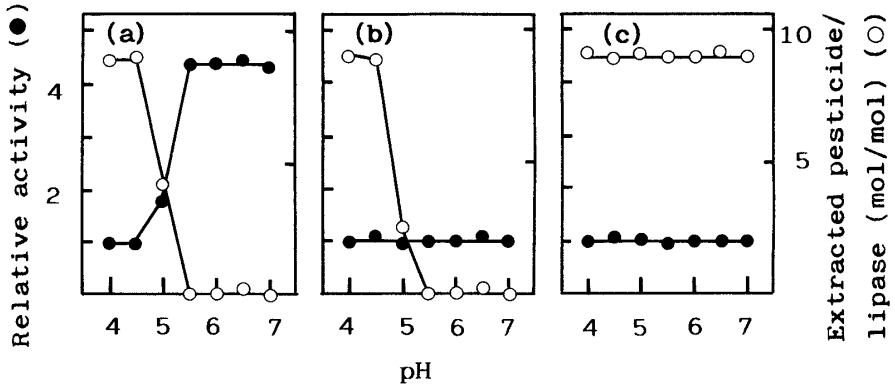

Fig. 4. Effect of pH on the Dissociation

(a) DDT. (b) Dimic. (c) Kelthane.

●, relative activity measured at pH 6.0 after removal of free pesticide by isopropyl ether treatment at a given pH shown in the figures; ○, molar ratio of pesticide extracted with isopropyl ether to lipase.

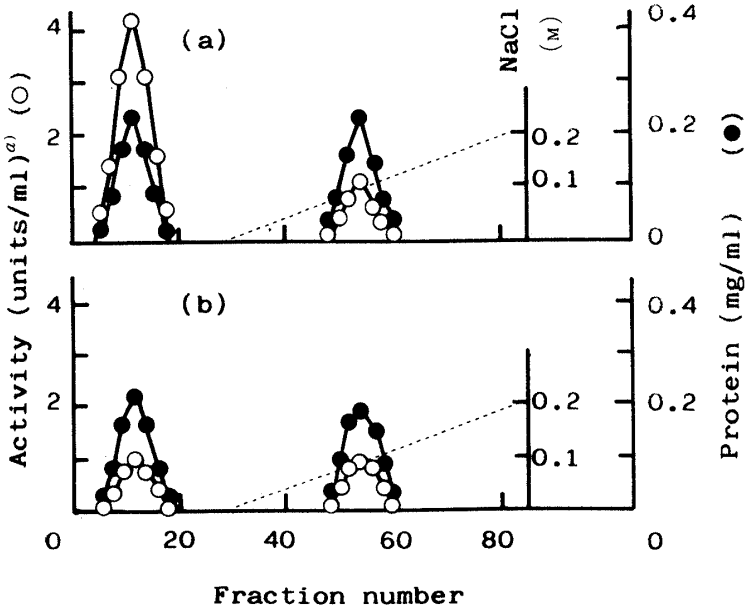

Fig. 5. CM-Sephadex C25 Column Chromatography of an Incubation Mixture of Pesticide and Lipase at a Molar Ratio of 5.5

(a) DDT. (b) Dimic.
○, activity; ●, protein measured by the method of Lowry *et al.*[9] using bovine serum albumin as a standard; ----, NaCl concentration.
a) One unit of activity releases of 1 μmol of fatty acid per minute under the assay conditions used.

respectively. With DDD and DDE, very similar results were obtained.

In the case of dimic, a mixture of dimic and lipase at the ratio of 5.5 was also resolved into two peaks each having the same specific activity as the intact lipase, as shown in Fig. 5b. On isopropyl ether treatment at pH 4.0, the molar ratios of dimic to lipase were found to be 9 : 1 for the first peak and 2 : 1 for the second peak, indicating the formation of E-DIM_9 and E-DIM_2 as in the case of DDT. This was confirmed by comparison of the elution profiles of E-DIM_9 and E-DIM_2 each prepared separately. With dichlorobenzophenone, a very similar result was obtained.

These findings imply one-step formation of 9 : 1 pesticide–lipase complex from 2 : 1 complex, supporting the previous hypothesis that E-DDT_2 changes directly into E*-DDT_9 without the formation of intermediate complexes, E-DDT_{3-8}.[4] Overlapping of the peaks of intermediate complexes on the peak of E-DDT_2 or E*-DDT_9 would affect the ratio of DDT to lipase.

With kelthane, however, only a single peak having the same elution volume and specific activity as the intact lipase was obtained.

In all the cases above described, protein in both peaks migrated each as a single protein band in polyacrylamide gel electrophoresis. The 2 : 1 complexes exhibited the same mobility as the intact lipase and the 9 : 1 complexes migrated more slowly than the intact lipase, as described previously (figure not shown).[4]

Discussion

Rh. delemar C-lipase is a hydrophobic protein with a molecular weight of 41300[10] or 45000.[3b] The average hydrophobicity of this lipase was 1270 cal/residue (the usual values lie between 1000 and 1100 cal/residue) as calculated from the amino acid composition.[10] The subunit structure is unknown, as far as we are aware. Treatment with phospholipids enhances

the activity towards synthetic lipoprotein[3] and human serum lipoprotein.[11] The 5:1 phosphatidylcholine–lipase complex, which was obtained on removal of excess phosphatidylcholine from the incubation mixture of lipase and phosphatidylcholine by extraction with a solvent mixture of isopropyl ether and *n*-butanol, was found to have a lower α-helical content and a lower isoelectric point compared with the native values.[3a,b] The phosphatidylcholine–lipase complex had a larger maximum velocity towards a synthetic substrate, while the Michaelis constant remained unchanged, and the activation was interpreted as arising from local structural change of the catalytic site caused by phosphatidylcholine.[3c]

The binding of DDT, DDE and DDD to lipase is very tight. No rounding-off in the sharp broken titration curves was observed even at 4×10^{-10} M lipase concentration (two orders of magnitude smaller than the concentration used in the usual lipase assay), indicating that the binding constant of 2:1 complex to form 9:1 complex is larger than 1.6×10^{11} M^{-1}, which was estimated, considering the error (<5%) in the enzyme assay. As to the high affinity binding of organochlorine to proteins, the binding constant of 2.68×10^{8} M^{-1} was reported for the binding of dieldrin to protein(s) in the "first protein peak" of cockroach hemolymph.[12]

The high affinity binding of the pesticide enabled us to observe the outstanding feature of the cooperative binding sites on this lipase, the first-binding pesticide governing the ensuing complex formation and activation, and the stepwise formation of pesticide–lipase complexes.

Interestingly, the 1:1 pesticide–lipase complexes govern the ensuing complex formation and activation of the lipase. The 1:1 complexes of all pesticides in group (1) bind any pesticide used here to form mixed 9:1 complexes with elevated activity towards tripropionin. On the other hand, the 1:1 complex of dimic, which belongs to group (2), does not bind any pesticide other than dimic itself, a strict negative cooperativity being operative.

The above findings indicate the importance of the sequence of addition of different pesticides as well as the importance of the 1:1 complexes. A similar phenomenon was previously observed.[4] That is, this lipase binds aldrin and DDT to form an "aldrin-activated" lipase (supposedly 2:7:1 DDT–aldrin–lipase complex) with a relative activity of 16 (the relative activity of 7:1 aldrin–lipase complex) when a mixture of DDT and aldrin was added to intact enzyme, whereas the lipase forms a "DDT-activated" lipase (supposedly 9:2:1 DDT–aldrin–lipase complex) with a relative activity of 4.4 (the relative activity of 9:1 DDT–lipase complex) when a mixture of DDT and aldrin was added to a preincubation mixture of DDT and lipase at 2:1 molar ratio.

In the present study, the ligand has neither charges nor groups available for hydrogen-bonds, and it is clear that this hydrophobic enzyme protein binds the hydrophobic ligand molecules mainly through hydrophobic interaction. The situation is, however, more complicated. It is very likely that the formation of 1:1 DDT–lipase complex governs the ensuing complex formation and activation, and the formation of 2:1 DDT–lipase complex causes the slow appearance of seven further binding sites, with slow conformational change. This indicates that the binding cannot be attributed simply to the hydrophobic character of the protein and ligand.

The possible mechanism of the binding of pesticide to *Rh.* lipase may be represented as shown in Chart 5 for DDT, where E′-DDT_2 is a precursor complex which changes into E-DDT_2, and v_1, v_2, v_3 and v_4 are the rates of transformation. The assumption of the slow formation of E-DDT_2 from the precursor complex, E′-DDT_2, and the very rapid formation of E*-DDT_9 from E-DDT_2 as compared with that in any other step is necessary to explain the

$$\mathrm{E} \xrightarrow{v_1} \text{E-DDT} \xrightarrow{v_2} \text{E'-DDT}_2 \xrightarrow{v_3} \text{E-DDT}_2 \xrightarrow{v_4} \text{E*-DDT}_9$$

Chart 5

experimental results, as discussed previously.[4] In addition, the exclusive formation of E-DDT prior to the formation of E-DDT_2 requires that v_1 is much faster than v_2. The order of rates of transformation should be $v_4 > v_1 > v_2 > v_3$, therefore.

Concerning the slow transformation of E′-DDT_2 to E-DDT_2, the results obtained by portionwise addition of DDT, which led us to the concept of exclusive 1 : 1 complex formation prior to 2 : 1 complex formation, is also suggestive for studies on the interaction between protein and ligand in that the experimental result may be dependent on how the reactants are mixed. Actually, about 1.25-fold activation was observed when 2 nmol of DDT was added to 1 nmol of lipase during 6 s (the preincubation time required for reproducible results), while no activation occurred when DDT was added rapidly.

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References and Notes

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