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ROLES OF TRYPTOPHAN RESIDUES ON THE *RHIZOPUS DELEMAR* LIPASE ACTIVITY: CHEMICAL MODIFICATION IN A WATER–OLIVE OIL EMULSION

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SUMMARY

1. The molecular weight of the lipase (EC 3.1.1.3) purified from *Rhizopus delemar* was 41 300. The average hydrophobicity, calculated from the results of amino acid analysis, was high. This suggests that a hydrophobic region of the lipase may play an important role in the formation of the enzyme–triglyceride complex (ES complex).

2. The tryptophan residues of the lipase were modified by 2-hydroxy-5-nitrobenzyl bromide (soluble in olive oil) and/or dimethyl-(2-hydroxy-5-nitrobenzyl) bromide (soluble in water) using the water–olive oil emulsion. It was found that one residue, modified from the olive oil side at the interface of the emulsion, was essential to the lipase activity and another one, modified from either the water or olive oil side might be a binding site. These two residues were buried in the lipase molecule unless the substrate was added, and were exposed to the enzyme surface when the enzyme–substrate complex was formed. Some speculation about the states of the other six residues is also presented.

INTRODUCTION

A lipase reaction system is considered to be a valuable model to study interactions between lipids and proteins. The lipases have been isolated from pancreas[1] and various microorganisms[2]. The structure and function of pancreatic lipase have been studied to elucidate the reaction mechanism of the hydrolysis of water-insoluble triglycerides. Desnuelle[2] pointed out that lipase acts at the interface of the triglyceride–water emulsions. Recently, it was shown, with a chemical modification method by Desnuelle and co-workers[3,4], that a serine residue was involved in and a histidine residue was probably involved in the active center of pancreatic lipase.

A chemical modification method has been used in reaction systems of water-soluble enzymes and water-soluble substrates as an useful probe of structure and function of enzymes. An additional use for this method is expected in the lipase

reaction system. An essential residue to the lipase action at the interface of the emulsion may be selectively modified by chemical reagents soluble in triglycerides.

In this paper the chemical modifications of tryptophan residues of *Rhizopus delemar* lipase by both a water-soluble reagent and a triglyceride-soluble reagent and the roles of tryptophan residues on the lipase activity are described.

MATERIALS AND METHODS

Reagents

Olive oil, dinitrofluorobenzene, *p*-chloromercuribenzoate and phenylmercuric chloride were purchased from Nakarai Chemicals Co. Ltd, trinitrobenzene sulfonate, from Tokyo Kasei Co. Ltd, and 2-hydroxy-5-nitrobenzyl bromide, from Sigma Chemical Co. Dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide was prepared from 2-hydroxy-5-nitrobenzyl bromide and dimethyl sulfide as described by Horton and Tucker[5].

Partially purified lipase from *Rh. delemar* was supplied by Tanabe Seiyaku Co. Ltd.

Enzyme assay

The reaction mixture contained 2 ml of olive oil, 5 ml of 0.1 M acetate buffer (pH 5.6) and 1.0 ml of the lipase solution in a L-shape test tube (diameter, 2.0 cm; height, 11.0 cm; width, 7.5 cm). The reaction was started by shaking the test tube (2 Hz, amplitude of 5.0 cm) in a water-bath shaker. The lipase showed the maximum activity under this shaking condition (see Fig. 7). After the incubation at 30 °C for 20 min, the reaction was stopped by the addition of 40 ml ethanol. Fatty acid released from olive oil was titrated with 0.05 M NaOH. An endpoint was determined with the use of an electrometric pH apparatus. In this assay system, deviation in shape and size of the test tubes may cause an experimental error. The test tubes in which the lipase showed the closest activities were selectively used.

Purification procedure of the lipase

Rhizopus delemar lipase was purified as described by Fukumoto *et al.*[6]. The presence of three kinds of lipase in *Rh. delemar* was reported by Tsujisaka[7]. Two minor components were excluded in sulfoethyl-Sephadex chromatography step. The dark brown color of this enzyme preparation was removed by the following procedure. To the enzyme in 0.1 M acetate buffer (pH 4.5), 0.6 vol. of 1% sodium alginate (pH 4.3) was added. The solution was allowed to stand overnight at 4 °C. The precipitate containing the lipase and alginate was dissolved in 0.2 M sodium acetate. To the solution, 0.05 vol. of 20% barium acetate, 0.1 vol. of acetone and 0.02 vol. of 20% basic lead acetate were added. Alginate was precipitated by centrifugation and discarded. To the supernatant, solid $(NH_4)_2SO_4$ was added (0.75 satn). The purified lipase was used on each experiment after removal of the salts by dialysis or gel filtration.

Chemical modification

The chemical modification procedures using water-soluble or olive oil-soluble reagents were carried out under the same conditions with respect to test tubes and

shaking as those of the enzyme assay. Experiments were performed under the following three conditions.

(1) The lipase was modified by water-soluble reagents in a buffer solution (8.0 ml) without any addition of olive oil. This modification in water solution is designated "*W*". This term is used as *W* modification, a modification under *W* condition, and so on.

(2) The lipase was modified by the water-soluble reagents in the olive oil emulsion. The emulsion was formed by shaking from 2 ml of olive oil and 6 ml of buffer solution containing the lipase. The lipase bound by the olive oil should be modified from the water side at the interface. This modification in the emulsion from the water side is designated "E_w".

(3) The conditions were the same as in E_w except that the water-soluble reagents were replaced by the oil-soluble reagents. The lipase bound by the olive oil should be modified from the oil side. This is designated "E_o".

Concentrations of chemical modifiers under the E_w and E_o conditions represent those in water and olive oil, respectively.

After the lipase was modified under these three conditions, a part of the lipase solution was withdrawn, and the enzyme activity and the amount of modified amino acid residue were determined. In the E_w and E_o modification the emulsion was separated into water and olive oil phases by centrifugation at 3000 rev./min for 5 min.

The amount of modified residue was determined as follows. Measurement of the sulfhydryl group of the lipase was carried out by the addition of a *p*-chloromercuribenzoate solution in the presence of or in the absence of 6 M urea. The amount of sulfhydryl group modified was calculated on the basis of the molar extinction coefficient at 250 nm, 7600 (see ref. 8). The amino groups were modified by dinitrofluorobenzene or trinitrobenzene sulfonate. The amount of dinitrophenylated amino group was determined in 1 M HCl from an absorbance at 350 nm ($\varepsilon = 1.48 \cdot 10^4$/M per cm) (see ref. 9), and that of the trinitrophenylated amino group, in 4 M urea and 0.5 M HCl from an absorbance at 344 nm ($\varepsilon = 1.09 \cdot 10^4$/M per cm) (see refs 10 and 11). The tryptophan residues of the lipase were modified by 2-hydroxy-5-nitrobenzyl bromide or dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide. The 2-hydroxy-5-nitrobenzyl lipase was precipitated by the addition of trichloroacetic acid (final concentration, 3%). The precipitate was dissolved in 0.1 M NaOH. The amount of tryptophan modified was determined from an absorbance at 410 nm ($\varepsilon = 1.8 \cdot 10^4$/M per cm) (see ref. 12).

Amino acid analysis

The lipase was hydrolyzed in 6 M HCl at 110 °C for 22 or 70 h. The procedure of each hydrolysis was performed in a duplicate manner. Amino acid analyses were carried out with a Hitachi amino acid autoanalyzer Model KLA-3.

The tryptophan content was determined spectrophotometrically by the method of Goodwin and Morton[13].

Ultracentrifugation

Ultracentrifugal analyses were carried out with a Beckman Model E analytical ultracentrifuge. A Schlieren optical system was used in the sedimentation experiment and a Rayleigh interference system, in the equilibrium centrifugation experiment.

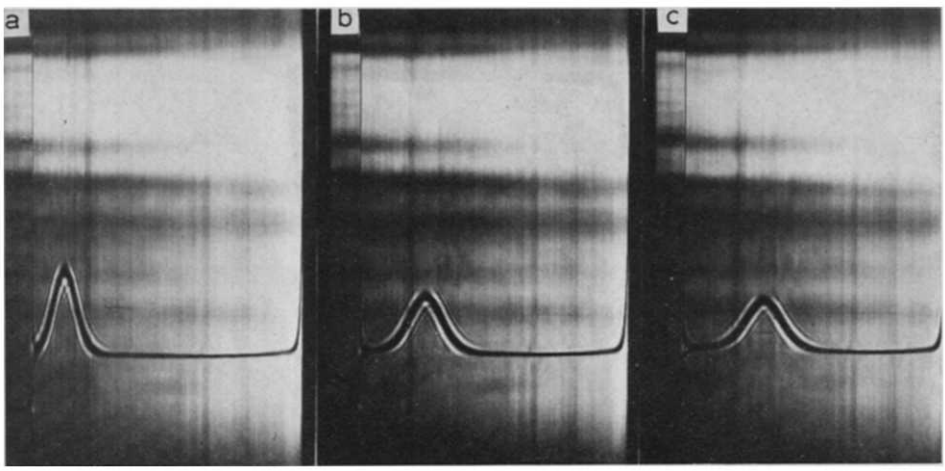

Fig. 1. Sedimentation pattern of *Rhizopus delemar* lipase. The ultracentrifugations were performed with 0.6% of the enzyme solution (10 mM acetate buffer (pH 5.0)) at 18 °C. Time after reaching maximum speed (60 000 rev./min): a, 39 min; b, 63 min; c, 87 min.

RESULTS

Molecular weight

Homogeneity of the purified lipase preparation was proved by an analytical ultracentrifugation and an acrylamide gel electrophoresis as shown in Figs 1 and 2.

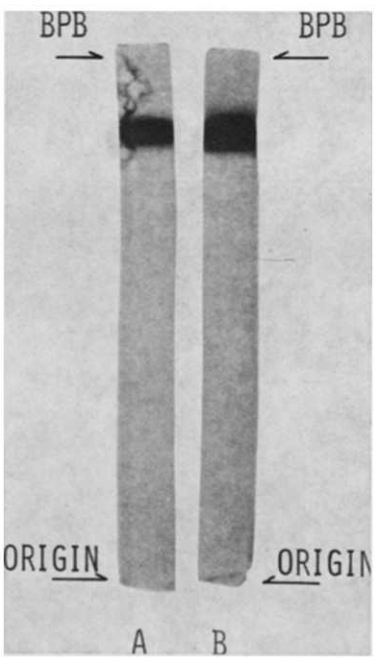

Fig. 2. Acryl amide gel electrophoresis. After electrophoresis for 90 min, the protein was stained by amido black 10B. The amount of enzyme: A, 60 μg; B, 100 μg. BPB, bromophenol blue.

The molecular weight was determined by means of the meniscus depletion method of Yphantis[14]. An equilibrium centrifugation was carried out with 0.02, 0.03 and 0.04% solutions of the lipase. The plots of the logarithm of the fringe displacements *versus* $(r)^2$ were linear. Fig. 3 presents data obtained from the experiment with the 0.03% enzyme solution. The average molecular weight calculated from the slopes of the plots was 41 300. The calculation was based on an assumption that a partial specific volume of the lipase was 0.75.

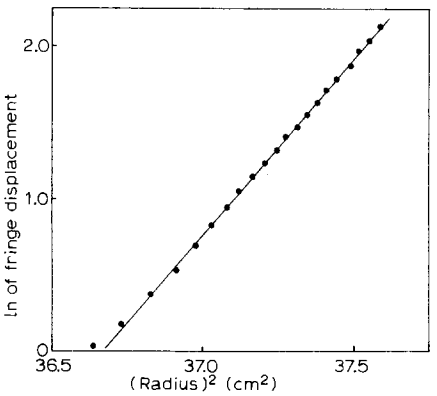

Fig. 3. Equilibrium centrifugation. The equilibrium centrifugation was carried out with a rotor speed of 32 000 rev./min at 17 °C. The lipase concentration was 0.03% in 50 mM phosphate buffer (pH 6.0).

Molecular extinction coefficient

The turbidimetric method[15,16] was used to relate protein concentration to absorbance of the lipase at 280 nm. Crystalline bovine serum albumin dried under a reduced pressure over P_2O_5 was used as a standard protein. The molecular extinction coefficient of the lipase at 280 nm was $7.5 \cdot 10^7$ cm²/mole.

Amino acid composition

The results of amino acid analysis are represented in Table I. The average hydrophobicity proposed by Bigelow[17] was calculated with the ΔF value reported by Tanford[18]. The average hydrophobicity of the lipase was 1270 cal/residue. It was shown by Bigelow[17] that the most proteins have average hydrophobicities between 1000 and 1100 cal/residue. The lipase value was relatively high as expected from the fact that the substrate of this enzyme is insoluble in water.

Effects of the oil-soluble and the water-soluble chemical modifiers

The lipase was chemically modified under the three conditions, W, E_w and E_o. Table II represents the results of the modifications of the cysteine, lysine and tryptophan residues.

A distinctive difference in the inactivation of the lipase was only observed in the case of the modification of the tryptophan residue using the W, E_w and E_o conditions.

Lysine residues seem not to have any significant importance on the lipase activity.

TABLE I

AMINO ACID COMPOSITION OF *Rhizopus delemar* LIPASE

Amino acid	*Residue/mole of enzyme*
Aspartic acid	30
Threonine*	19
Serine*	15
Glutamic acid	45
Proline	17
Glycine	9
Alanine	25
Valine**	22
Methionine	2
Isoleucine**	8
Leucine	33
Tyrosine	30
Phenylalanine	15
NH_3*	51
Lysine	33
Histidine	9
Arginine	13
Half-cystine	6
Tryptophan***	8

* Obtained from the extrapolation of the linear relation between the amount of residue and the hydrolysis time.

** Value of 70 h of hydrolysis.

*** Estimated spectrophotometrically.

TABLE II

CHEMICAL MODIFICATION OF THE LIPASE UNDER THE W, E_w AND E_0 CONDITIONS

Sulfhydryl group: the lipase activity was measured after 12.5 μM of the enzyme in 0.1 M phosphate buffer (pH 7.0) was modified by 10 mM sulfhydryl reagents at 30 °C for 1 h. The amount of sulfhydryl group was determined by *p*-chloromercuribenzoate titration with 14 μM of the lipase in 0.1 M phosphate buffer (pH 7.0). Amino group: the lipase (5 μM) was modified in 0.1 M phosphate buffer (pH 7.0) by 2.0 mM of the modifying reagents for 5 h at 25 °C. Tryptophan residue: concentrations of the modifying reagents were 10 mM. Modifications were carried out in 0.1 M acetate buffer (pH 5.0) with 12.5 μM of the lipase for 4 h at 30 °C. The residual activity and the amount of residue modified were determined as described in Materials and Methods.

Residue	*Modifying reagent*	*Residual activity (%)*			*Mole of residue/mole of enzyme*		
		W	E_w	E_0	W	E_w	E_0
Sulfhydryl group	*p*-Chloromercuribenzoate	97	93	—	0	N.D.	—
	Phenylmercuric chloride	—	—	98	—	—	N.D.
Amino group	Trinitrobenzene sulfonate	85	96	—	6.8	1.1	—
	Dinitrofluorobenzene	—	—	76	—	—	5.0
Tryptophan residue	Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide	95	50	—	5.0	3.9	—
	2-Hydroxy-5-nitrobenzyl bromide	—	—	0	—	—	3.5

N.D., not determined.

The results of the modification by the sulfhydryl reagents indicate that a cysteine residue does not exist on the surface of the native lipase molecule. The experiment of *p*-chloromercuribenzoate titration was also carried out in 6 M urea. The presence of a sulfhydryl group was not detected in the urea-denatured lipase. These results suggest that the six half-cystine residues determined by the amino acid analysis should be attributed to three disulfide linkages.

Modifications of the tryptophan residues

As suggested in Table II, the tryptophan residue(s) may play an important role on the catalytic activity or on the maintenance of a spatial conformation of the lipase. It is supposed from the value of the average hydrophobicity that a hydrophobic region of the lipase may have a large contribution to the formation of the enzyme–triglyceride complex. A tryptophan residue, the amino acid of the highest hydrophobicity, may be a marker of a structural change in a hydrophobic region of the lipase. More detailed experiments chemically modifying the tryptophan residues were carried out under the W, E_w and E_0 conditions.

The distributions of both 2-hydroxy-5-nitrobenzyl bromide and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide between water and olive oil was determined by measuring the absorbance of the reagents at 410 nm under the same conditions as E_w or E_0. The partition constants of 2-hydroxy-5-nitrobenzyl bromide (water/oil) and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide (oil/water) were 0.01 and 0.006, respectively. These low values of the partition constants make it unlikely that 2-hydroxy-5-nitrobenzyl bromide, dissolved in water, or dimethyl (2-hydroxy-5-nitrobenzyl)sulfonium bromide, dissolved in olive oil, may have some influence on the results of the E_w or E_0 modification.

Figs 4a and 4b represent the time-courses of the loss of the lipase activity, and the plots of the residual enzyme activity *versus* the amount of 2-hydroxy-5-nitrobenzyl tryptophan per mole of the lipase, respectively.

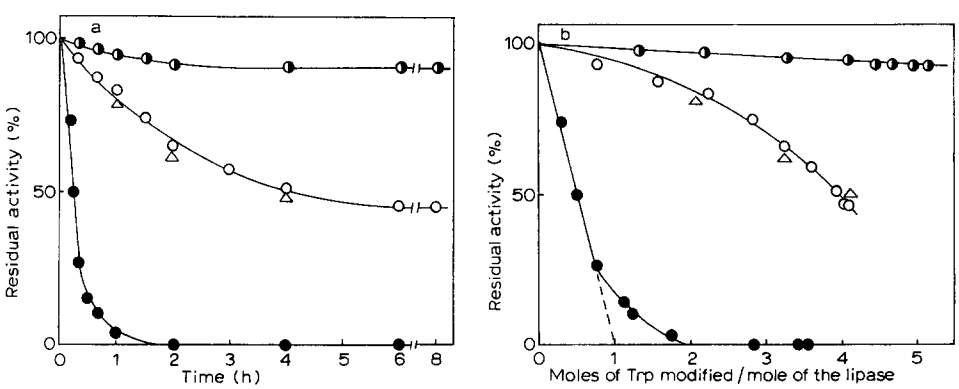

Fig. 4 (a) and (b). Tryptophan residues modified under the W, E_w and E_0 conditions. The lipase (12.5 μM) in 0.1 M acetate buffer (pH 5.0) was modified by 10 mM 2-hydroxy-5-nitrobenzyl bromide or 10 mM dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide at 30 °C. Other conditions were the same as described in Materials and Methods. ◑, W modification; ○, E_w modification; ●, E_0 modification; △, E_w modification after 2-h incubation of the lipase (in 6 ml of 0.1 M acetate buffer (pH 5.0) with olive oil (2 ml)) at 30 °C with shaking (2 Hz, 5-cm amplitude).

About five moles of the tryptophan residues per mole of the lipase were modified under the W condition without any remarkable loss of the enzyme activity.

On the other hand, the lipase was rapidly inactivated by the E_0 modification. About four residues were modified under the E_0 condition.

Under the E_W condition, about four residues were modified at a relatively slow rate, and the lipase activity was decreased to about 50% of the native enzyme. The products released from olive oil by the lipase action may influence the E_W modication, because under this condition a rapid inactivation of the lipase does not occur. In order to clarify this problem, the lipase was modified under the E_W condition after a 2-h preincubation. The preincubation condition was the same as the E_W modification except that dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide was not added. Distinctive effects of the products on the E_W modification were not observed as shown by the triangles in Fig. 4.

In the E_0 modification, the value of one tryptophan residue per mole of the lipase was obtained by extrapolating the linear relation between the loss of the enzyme activity and the amount of tryptophan modified as shown in the dotted line in Fig. 4b.

The essential tryptophan residue

The presence of one tryptophan residue essential to the lipase activity is suggested in Fig. 4b. It may be due to the presence of non-essential tryptophan residues that the enzyme activity is not completely inactivated by the E_0 modification of one residue.

The E_0 modification was carried out after the non-essential tryptophan residues were previously modified under the W condition. The lipase activity was almost completely lost by the E_0 modification of one tryptophan residue as shown in Fig. 5. This result indicates that one tryptophan residue, which is in contact with olive oil, is essential to enzyme activity. Fig. 5 also shows that two residues are modified under the E_0 condition after the W modification.

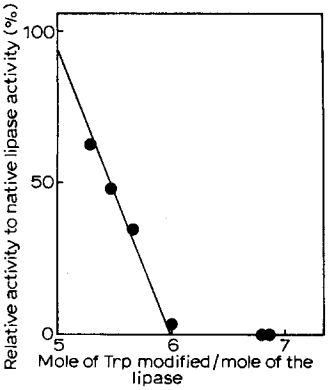

Fig. 5. The relationship between the residual lipase activity and the amount of tryptophan modified under the E_0 condition after W modification. The lipase (12.5 μM) was previously modified for 5 h by 10 mM dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide under the W condition. After a dialysis of the solution against 0.1 M acetate buffer (pH 5.0) for 12 h at 4 °C, the lipase was again modified by 10 mM 2-hydroxy-5-nitrobenzyl bromide under the E_0 condition.

Partial inactivation by E_w modification

The number of tryptophan residues which cause the partial inactivation due to the E_w modification are not clarified in Fig. 4b.

The lipase was modified under the E_w condition after W modification. Only one residue was modified, and the linear relation between the residual enzyme activity and the amount of tryptophan modified was observed as shown in Fig. 6. These results indicate that one tryptophan residue caused the partial inactivation of the lipase by E_w modification.

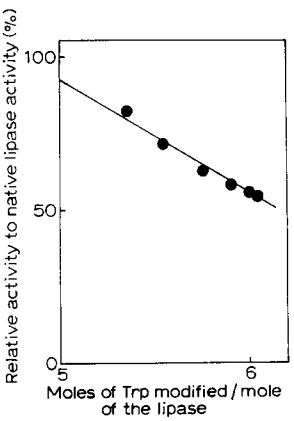

Fig. 6. Modification of the tryptophan residue under the E_w condition after W modification. The lipase (12.5 μM) was previously modified under the W condition as described in the legend of Fig. 5. To the W-modified enzyme solution (6 ml), 2 ml of olive oil was added. E_w modification was carried out without any further addition of dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide. Other conditions of E_w modification was the same as described in Materials and Methods.

The lipase activity modified under the E_w condition was assayed by varying the frequency of shaking. The interface area of the olive oil emulsion should be increased with the increase of the shaking frequency. The native lipase activity was increased and reached a maximum with the increase of the shaking frequency as indicated by the filled circles in Fig. 7. The ratio of E_w-modified lipase activity to the native enzyme was increased by the increase of the shaking frequency. It is likely that the partial inactivation by the E_w modification is mainly due to a decrease of affinity of the lipase for olive oil. The maximum activity of E_w modified lipase was not able to be measured because of the limitation of the shaking incubator. Thus it is not obvious if some change in thes patial conformation of the lipase is brought about by E_w modification.

Overlapping of the tryptophan residues modified under W, E_w and E_o conditions

Table III is a summary of data in Figs 4–6, and of some additional experiments under the conditions of combinations of W, E_w and E_o.

The results of Expts 1–3 indicate that the sum of tryptophan residues modified under each condition is thirteen. The eight tryptophan residues were detected in the lipase as shown in Table I. These results indicate that the residues modified under each condition overlap. The overlapping number can be calculated by subtracting the final number of the stepwise-modified residues (*e.g.* six in W and E_w) from the

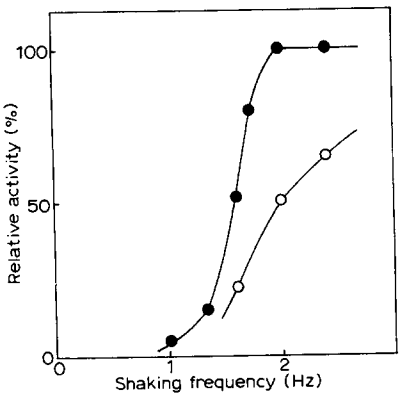

Fig. 7. The lipase activity partially inactivated by E_w modification. After the lipase (12.5 μM) was modified for 4 h under the E_w condition, the emulsion was centrifuged at 3000 rev./min for 5 min. The lipase activity of the water layer was measured as described in Materials and Methods. ●, native lipase; ○, E_w-modified lipase.

sum of the independently-modified residues (*e.g.* nine in W and E_w). It is suggested by the results of Expts 1–6, that the overlapping numbers of W:E_w, W:E_0, E_w:E_0 are three, two and one, respectively.

The modification of the three steps ($W \rightarrow E_w \rightarrow E_0$) were carried out as shown in Expt 7. The number of residues finally modified in Expt 7 agreed well with the value which is calculated by subtracting the sum of the overlapping residues (six) from the sum of the independently-modified residues (13). Each modification of one

TABLE III

SUMMARY OF THE MODIFICATIONS OF TRYPTOPHAN RESIDUES

Results in Expts 1–5 are the data in Figs 4–6. Expt 6: the enzyme (13 μM) was modified for 5 h under the E_w condition. The reaction mixture was centrifuged at 3000 rev./min for 5 min, and 5 ml of the enzyme solution was withdrawn. The solution was dialyzed against 0.1 M acetate buffer (pH 5.0) for 12 h at 4 °C, and was filled up to 6 ml with the same buffer. The enzyme was again modified under the E_0 condition. The conditions of the E_w and E_0 modification were the same as in Table II. Expt 7: the conditions of $W \rightarrow E_w$ and $E_w \rightarrow E_0$ were the same as in Expts 4 and 6, respectively. Expt 8: the lipase (13 μM) was incubated with 6 M urea (pH 3.0) for 7 h at room temperature. To this enzyme solution, dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide was added to bring the final concentration to 10 mM. After a 5-h incubation at 30 °C, the amount of tryptophan modified was determined as described in Materials and Methods.
The values in the parentheses represent a nearest intergar.

Experiment	*Modification*	*Reaction time (h)*	*Number of tryptophan modified*	*Residual activity (%)*
1	W	8	5.1 (5)	95
2	E_w	8	4.1 (4)	48
3	E_0	6	3.6 (4)	0
4	$W \rightarrow E_w$	5 → 4	5.0 (5) → 6.0 (6)	95 → 49
5	$W \rightarrow E_0$	5 → 2	5.0 (5) → 6.9 (7)	95 → 0
6	$E_w \rightarrow E_0$	5 → 4	4.2 (4) → 6.7 (7)	48 → 0
7	$W \rightarrow E_w \rightarrow E_0$	5 → 4 → 2	5.0 (5) → 5.8 (6) → 7.0 (7)	95 → 50 → 0
8	W (urea)	5	7.8 (8)	—

tryptophan residue at the E_w and E_0 modification caused 50 and 100% inactivation of the lipase, respectively. This result proves that one tryptophan residue modified under the E_w condition, which causes the partial inactivation, is not identical with the one essential residue modified under the E_0 condition.

It is indicated in Expt 8 that about eight tryptophan residues are modified in urea-denatured lipase by dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide. This value agreed with the result in Table I. One tryptophan residue seems to be buried in the lipase molecule under the conditions of W, E_w and E_0.

DISCUSSION

Both reagents of 2-hydroxy-5-nitrobenzyl bromide[12,19] and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide[5] also react with a cysteine residue. The results of Table II and *p*-chloromercuribenzoate titration of the urea-denatured lipase suggest that the six half-cystine residues detected by the amino acid analysis correspond to the three cystine residues. Thus the chromophore of the 2-hydroxy-5-nitrobenzyl group introduced to the lipase is considered to be due to the selective modification of the tryptophan residues.

It was reported that the reaction of 2-hydroxy-5-nitrobenzyl bromide with tryptophan residues also gave a di-substituted product[12,20]. However, the stoichiometric relation between the amount of residue modified per mole of the lipase and the loss of the enzyme activity was shown in the E_0 and E_w modification (see Figs 5 and 6). The result of the modification of the urea-denatured enzyme agreed well with that of the amino acid analysis. As judged from the results reported by Barman and Koshland[12], the concentrations of the modifying reagents used in these experiments seem not to be so high as to give the di-substituted product, although a valid concentration of the reagent, which reacts with the tryptophan residues at the interface of the olive oil emulsion, is not clear. These make it very probable that almost all the tryptophan residues are modified by the reagents to give a mono-substituted product.

The eight tryptophan residues can be classified into five groups with respect to the states of a spatial conformation of the lipase molecule or to roles on the lipase activity as shown in Scheme I. Scheme I is obtained from Table III. The eight residues are numbered from No. 1 to No. 8 on the following assumptions ((A)–(D)). (A) The five residues modified under the W condition are Nos 1–5. (B) The overlapping three between the W and E_w modification are Nos 3–5. (C) The one modified under the E_w condition after W modification is No. 6. (D) The one modified under the E_0 condition after W and E_w modification is No. 7. Then, the overlapping two between the W and E_0 modification should belong to Nos 1 and 2, and the overlapping one between the E_w and E_0 modification, No. 6. No. 8 is modified only in the urea-denatured lipase. The first group (I), Nos 1 and 2, is non-essential to the lipase activity and is exposed to olive oil at the interface of the emulsion. The presence of these two residues which are modified under the W condition, but not under the E_w condition, may indicate that almost all the lipase molecules are bound by olive oil under the E_w condition, as expected from the fact that the lipase activity shows the maximum value under the shaking condition of E_w modification. The second (II), Nos 3–5, is also non-essential to the lipase activity and is exposed to water at the

SCHEME I

STRUCTURE AND FUNCTION OF THE TRYPTOPHAN RESIDUES

+ represent "modified".

No. of residue	*Group*	*Modification*				*Structure*	*Function*
		W	E_w	E_0	*W (urea)*		
1	I	+		+	+	Exposed to oil	Non-essential
2		+		+	+		
3	II	+	+		+	Exposed to water	Non-essential
4		+	+		+		
5		+	+		+		
6	III		+	+	+	Exposed to water and oil	(Binding site)
7	IV			+	+	Exposed to oil	Catalytic site
8	V				+	Buried	Not clear

interface of the emulsion. The third (III), No. 6, is in contact with both water and oil. This is consistent with the suggestion obtained from Fig. 7 that the binding of the lipase to olive oil may be inhibited by the modification of the No. 6 residue. The fourth (IV), No. 7, is essential to the catalytic activity of the lipase and is exposed to the oil. The residues of Nos 6 and 7 are considered to be exposed to the surface of the lipase molecule according to the formation of the enzyme–substrate complex, since these residues are not modified under the *W* condition, but are modified under the E_w and E_0 conditions. The fifth (V), No. 8, is buried in the lipase molecule under the *W*, E_w and E_0 condition.

It was reported by Desnuelle and co-workers[3,4] that a serine and possibly a histidine residue were involved in the active center of pancreatic lipase. In fact, the various hydrolytic enzymes are known to have a serine and a histidine residue on the active center. If an effective reagent is obtained, it may be meaningful to modify these residues of *Rh. delemar* lipase under the *W*, E_w and E_0 condition.

Maylie *et al.*[4] also showed that the essential serine of pancreatic lipase was modified only by the emulsified reagent. Although their observations do not necessarily provide information about a state of pancreatic lipase bound by the substrate, the results of the chemical modifications of both pancreatic and *Rh. delemar* lipase suggest that the essential residues buried in the enzyme molecule may be exposed to the enzyme surface by the formation of the enzyme–substrate complexes.

Chemical modification with the use of both oil-soluble reagents and water-soluble reagents is considered to be useful in the study of the structure and function of proteins which interact with lipids to play an important role on biological activities.

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REFERENCES

1 Sarda, L., Marchis-Mouren, G., Constantin, M. J. and Desnuelle, P. (1957) *Biochim. Biophys. Acta* 23, 264–274
2 Desnuelle, P. (1972) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn, Vol. 7, pp. 575–616, Academic Press, London and New York
3 Sémériva, M., Dufour, C. and Desnuelle, P. (1971) *Biochemistry* 10, 2143–2149
4 Maylíe, M. F., Charles, M. and Desnuelle, P. (1972) *Biochim. Biophys. Acta* 276, 162–175
5 Horton, H. R. and Tucker, W. P. (1970) *J. Biol. Chem.* 245, 3397–3401
6 Fukumoto, J., Iwai, M. and Tsujisaka, Y. (1964) *J. Gen. Appl. Microbiol.* 10, 257–265
7 Tsujisaka, Y. (1970) *Kagaku to Seibutsu* 8, 315–320
8 Boyer, P. D. (1954) *J. Am. Chem. Soc.* 76, 4331–4337
9 Porter, R. R. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 4, pp. 221–237, Academic Press, London and New York
10 Goldfarb, A. R. (1966) *Biochemistry* 5, 2570–2574
11 Sasaki, R., Sugimoto, E. and Chiba, H. (1971) *Biochim. Biophys. Acta* 227, 584–594
12 Barman, T. E. and Koshland, Jr. D. E. (1967) *J. Biol. Chem.* 242, 5771–5776
13 Goodwin, T. W. and Morton, R. A. (1946) *Biochem. J.* 40, 628–632
14 Yphantis, D. A. (1964) *Biochemistry* 3, 297–317
15 Bücher, T. (1947) *Biochim. Biophys. Acta* 1, 292–314
16 Rosenbloom, J., Sugimoto, E. and Pizer, L. I. (1968) *J. Biol. Chem.* 243, 2099–2107
17 Bigelow, C. C. (1967) *J. Theor. Biol.* 16, 187–211
18 Tanford, C. (1962) *J. Am. Chem. Soc.* 84, 4240–4247
19 Horton, H. R. and Koshland, Jr, D. E. (1965) *J. Am. Chem. Soc.* 87, 1126–1132
20 Kondo, Y. and Witkop, B. (1968) *J. Am. Chem. Soc.* 90, 3256–3258