Tryptophan Residues Involved in the Binding of Chlorinated Pesticides by *Rhizopus delemar* C-Lipase: Isolation of Tryptophan-Containing Peptides

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Tryptophan (Trp) residues involved in the binding of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 2,2-bis(4-chlorophenyl)ethane (DIM) and dichlorobenzophenone (DCBP) by *Rhizopus delemar* C-lipase were identified on the basis of knowledge of amino acid sequences around the Trp residues. Eight Trp- or modified Trp-containing peptides were isolated from each of 1:1, 2:1 and 9:1 pesticide-lipase complexes modified by 2-hydroxy-5-nitrobenzylation under three different conditions, and the peptides were identified.

The results confirm the previous tentative assignment of the Trp residues involved in the ligand binding, which was presented on the basis of comparison of their modification patterns.

Keywords *Rhizopus delemar*; lipase; chlorinated pesticide; ligand binding; hydroxynitrobenzylation; modification, tryptophan

Introduction

Rhizopus delemar C-lipase is a unique fungus lipase in that it binds strongly and stoichiometrically some chlorinated pesticides such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 2,2-bis(4-chlorophenyl)ethane (DIM), dichlorobenzophenone (DCBP) and aldrin.[1)] Although all three structurally related pesticides, DDT, DIM and DCBP are similar in that they are bound by the lipase to form stable 1:1, 2:1 and 9:1 pesticide-lipase complexes (E-pesticide, E-pesticide$_2$ and E-pesticide$_9$), the first bound ligand molecule affects the binding property of the lipase quite differently depending on the sort of pesticide. That is, E-DDT binds ligand, whether it is DDT, DIM or DCBP, to form a complex, E-DDT-pesticide$_8$ with 4.4 times the activity of intact lipase toward tripropionin as a water-soluble substrate, while E-DIM binds only DIM and E-DCBP binds only DCBP, without the change of enzyme activity.[1b)] This concept was supported by a modification study, in which the tryptophan (Trp) residues on the complexes of the above three pesticides behaved differently toward the modification (2-hydroxy-5-nitrobenzylation) depending on the sort of pesticide used as a ligand.[2)]

Selective modification of Trp residues by 2-hydroxy-5-nitrobenzylation under appropriate conditions of pH in the absence of sulfhydryl groups has been well established.[3)] The lipase possesses eight Trp residues and no sulfhydryl group on the single peptide protein with a molecular weight of 41300,[4)] whose primary structure has not been reported. Chiba *et al.*[4)] classified the eight Trp residues (T1 to T8) by 2-hydroxy-5-nitrobenzylation under three conditions: modification with water-soluble reagent (W modification, modification of exposed Trp residues), with the same reagent in olive oil emulsion (Ew modification, modification of Trp residues exposed to water in the presence of olive oil) and with fat-soluble reagent in the emulsion (Eo modification, modification of Trp residues exposed to oil). The modification pattern for native lipase is shown in Table I. Trp residues, T1 and T2, presumably located on a hydrophobic surface area, are modified by W and Eo. T3, T4 and T5, presumably located on a hydrophilic surface area, are modified by W and Ew. T6, presumably exposed to water and to oil when oil binds to lipase, is modified by Ew and Eo. T7, involved in the enzyme activity, was modified only by Eo. T8 is considered to be buried in the lipase molecule. All Trp residues (T1 to T8) are modified by W in the presence of 6 M urea.

In a previous study on the modification of pesticide-lipase complexes by 2-hydroxy-5-nitrobenzylation, we tentatively

TABLE I.[a)] Tentative Assignment of Modified Tryptophan Residues[b)] in Lipase Binding Chlorinated Pesticides

Lipase and pesticide-lipase complexes	W condition								Ew condition								Eo condition							
	N[c)]	T1	T2	T3	T4	T5	T6	T7	N[c)]	T1	T2	T3	T4	T5	T6	T7	N[c)]	T1	T2	T3	T4	T5	T6	T7
Native lipase	5	○	○	○	○	○	—	—	4	—	—	○	○	○	○	—	4	○	○	—	—	—	○	○
Lipase binding chlorinated pesticides																								
DDT-lipase complexes																								
E-DDT	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E-DDT$_2$	3	—	—	○	○	○	—	—	1	—	—	—	—	—	○	—	5	—	—	○	○	○	○	○
E-DDT$_9$	2	—	—	—	—	—	○	○	1	—	—	—	—	—	○	—	2	—	—	—	—	—	○	○
DIM-lipase complexes																								
E-DIM	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E-DIM$_2$	3	—	—	○	○	○	—	—	4	—	—	○	○	○	○	—	2	—	—	—	—	—	○	○
E-DIM$_9$	0	—	—	—	—	—	—	—	1	—	—	—	—	—	○	—	2	—	—	—	—	—	○	○
DCBP-lipase complexes																								
E-DCBP	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E-DCBP$_2$	4	—	○	○	○	○	—	—	4	—	—	○	○	○	○	—	3	—	○	—	—	—	○	○
E-DCBP$_9$	1	—	○	—	—	—	—	—	1	—	—	—	—	—	○	—	3	—	○	—	—	—	○	○

a) Summary of previous report.[2)] *b*) Open circles indicate modified Trp residues and horizontal lines indicate unmodified Trp residues. *c*) Number of modified residues rounded to the nearest integer.

assigned the Trp residues involved in the binding of the pesticides by the lipase on the basis of the classification by Chiba *et al.*,[4] assuming that the ligand binding is principally to protect the residue involved in the binding from the modification (Table I).[2] These modification studies have a drawback in that the rather speculative assignment of the Trp residues involving in binding must be abolished if the classification by Chiba *et al.* is found to be incorrect. The purpose of the present study was first to specify the Trp residues (T1 to T8) in terms of the amino acid sequence around them, and to confirm the previous assignment of Trp residues involved in the binding of pesticide.

Results

Peptides containing Trp residues, T1 to T8, were designated as T1P to T8P and the corresponding 2-hydroxy-5-nitrobenzylated (HNB) Trp residue-containing peptides were designated as HNB-T1P to HNB-T8P.

Allocation of Trp Residues, T1 to T8, to the Corresponding Trp-Containing Tryptic Peptide Fragments The reduced and S-carboxymethylated (RCM) native lipase was exhaustively digested with trypsin, and eight Trp-containing peptides (T1P to T8P) were separated from the digest by high performance liquid chromatography (HPLC) using a reversed-phase column at pH 2.0 as shown in Fig. 1a. Fractions were further purified by reversed-phase HPLC using an ammonia-alkaline eluant to give T1P to T8P. Their amino acid sequences as determined by the 4-*N*,*N*-dimethyl-aminoazobenzene-4′-isothiocyanate (DABITC) method[5] are shown in Fig. 2. The amino acid compositions as well as the sequences were different from each other.

Similarly, eight counterpart HNB-Trp-containing peptides (HNB-T1P to HNB-T8P) were obtained from the tryptic digest of a lipase that was fully modified by W modification in the presence of 6 M urea (Fig. 1b). Sequencing by the DABITC method was unsuccessful, as was reported for the Edman degradation of HNB-Trp-containing peptide from HNB-papain,[6] but each of HNB-T1P to HNB-T8P had the same amino acid composition as the corresponding T1P to T8P.

Native lipase which was modified by W, Ew and Eo was treated similarly to afford Trp- and HNB-Trp-containing peptides, and these were analyzed for amino acid composition. Considering the reactivity of Trp residues for the three modifications as described by Chiba *et al.*,[4] the peptides were allocated to Trp residues T1 to T8, respectively, as shown in Fig. 2. The allocation was arbitrary for T3, T4 and T5 as they exhibited the same reactivity for the modifications, and that for T1 and T2 will be described

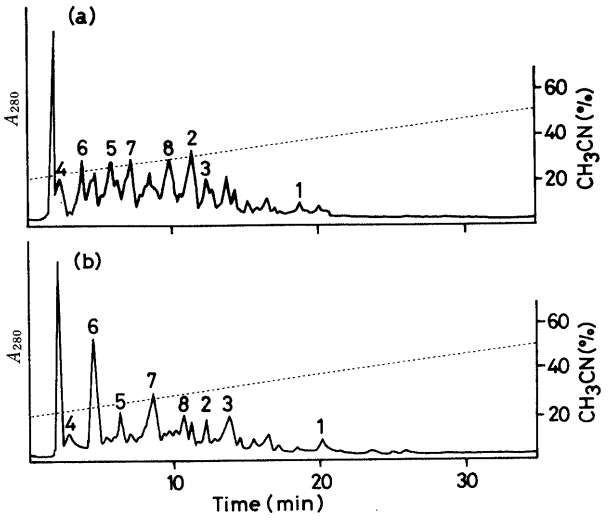

Fig. 1. HPLC of Tryptic Peptide Fragments from Native Lipase (a) and HNB-Lipase (b)

Peaks 1 to 8 contained T1P to T8P (a) or HNB-T1P to HNB-T8P (b), respectively.

T1P Phe–Phe–Trp–Lys
T2P Gly–Asn–Leu–Trp–Ala–Lys
T3P Leu–Trp–Met–Arg
T4P Gly–Gly–Trp–Ala–Arg
T5P Tyr–Trp–Lys
T6P His–Trp–Lys
T7P Trp–Pro–Tyr–Gly–Leu–Ser–Gly–Lys
T8P Trp–Met–Asp–Phe–Lys

Fig. 2. Amino Acid Sequences of Trp-Containing Peptides from *Rh. delemar* C-Lipase

Table II. Yields of Trp-containing peptide

Complex	Trp-containing peptides							
	T1P	T2P	T3P	T4P	T5P	T6P	T7P	T8P
Native	72.6	70.2	64.4	60.8	69.9	71.8	66.6	62.3
W	56.3[a]	63.2[a]	58.5[a]	58.3[a]	63.4[a]	63.5	60.6	59.9
Ew	66.6	65.4	62.5[a]	61.5[a]	60.8[a]	68.8[a]	60.6	63.3
Eo	69.5[a]	72.2[a]	67.2	62.0	71.2	71.8[a]	69.3[a]	65.5
W (Urea)	70.5[a]	68.7[a]	61.8[a]	62.1[a]	61.1[a]	68.8[a]	63.4[a]	64.5[a]
E-DDT								
W	63.5	72.5[a]	68.5[a]	77.3[a]	68.4[a]	70.8	67.6	54.9
Ew	71.8	70.4	66.5[a]	55.3[a]	68.8[a]	78.0[a]	59.6	68.9
Eo	69.5	77.7[a]	60.5	61.8	62.8	71.8[a]	62.8[a]	68.9
E-DDT_2								
W	51.8	60.2	70.7[a]	71.5[a]	60.8[a]	71.2	55.5	60.9
Ew	66.5	68.7	51.4	49.8	71.8	68.0[a]	52.2	65.4
Eo	69.5	72.4	65.4[a]	63.2[a]	62.1[a]	76.5[a]	55.2[a]	65.4
E-DDT_9								
W	77.6	66.5	65.7	51.8	74.3	72.3[a]	56.7[a]	67.8
Ew	61.2	55.4	68.8	80.2	53.4	58.7[a]	54.2	51.7
Eo	55.4	53.2	60.4	64.8	62.7	62.7[a]	62.2[a]	63.4
E-DIM								
W	55.5	66.6[a]	61.5[a]	65.3[a]	70.0[a]	70.1	69.9	61.8
Ew	65.9	65.9	70.2[a]	70.8[a]	60.8[a]	67.0[a]	48.7	70.8
Eo	70.2	70.7[a]	70.6	70.0	64.8	78.8[a]	70.5[a]	69.5
E-DIM_2								
W	70.5	69.8	71.5[a]	72.5[a]	70.8[a]	70.8	68.4	63.9
Ew	62.8	69.7	68.8[a]	66.6[a]	70.8[a]	74.2[a]	72.2	69.9
Eo	69.6	73.5	66.5	64.3	63.5	72.5[a]	65.3[a]	65.4
E-DIM_9								
W	77.6	66.5	65.7	51.8	74.3	72.3	56.7	67.8
Ew	69.3	61.8	65.5	68.6	65.8	62.5[a]	65.5	70.7
Eo	50.8	73.8	71.4	68.5	73.3	66.6[a]	67.2[a]	69.9
E-DCBP								
W	78.6	70.5[a]	72.1[a]	68.3[a]	71.1[a]	70.8	70.0	69.8
Ew	66.4	69.9	68.7[a]	65.8[a]	70.0[a]	68.8[a]	61.8	73.8
Eo	70.1	70.2[a]	69.5	72.2	68.8	72.4[a]	71.3[a]	69.5
E-$DCBP_2$								
W	71.1	69.4[a]	71.0[a]	71.8[a]	66.8[a]	59.8	63.4	63.9
Ew	61.1	73.7	60.5[a]	67.7[a]	72.2[a]	70.8[a]	72.0	71.1
Eo	70.5	72.6[a]	69.8	68.8	61.5	68.4[a]	60.8[a]	63.3
E-$DCBP_9$								
W	71.5	71.8[a]	67.7	68.8	65.9	71.5	67.8	60.5
Ew	67.3	68.0	70.5	68.8	64.4	66.4[a]	67.2	71.1
Eo	65.8	76.3[a]	72.2	72.0	72.0	76.0[a]	70.8[a]	73.3

a) HNB-containing peptides.

in Discussion.

Trp- and HNB-Trp-Containing Peptides from Pesticide-Lipase Complexes The 1 : 1, 2 : 1 and 9 : 1 pesticide-lipase complexes were modified by W, Ew and Eo. Eight Trp- or HNB-Trp-containing peptides were obtained from each Trp-modified complex when treated similarly. Since it was difficult to identify Trp- and HNB-Trp-containing peptides merely from their retention time, the isolated peptides were analyzed for amino acid composition after purification by HPLC and allocated to T1P to T8P and HNB-T1P to HNB-T8P. The results are shown in Table II. Comparison of the results in Tables I and II indicates that the results in Table II support the previous assignment of Trp residues involved in the binding shown in Table I.

Discussion

It was previously reported that, although the first bound pesticide molecule affects the binding property of the lipase in three different directions depending on the sort of pesticide,[1] the binding in E-DDT, E-DIM and E-DCBP involves the same Trp residue (T1).[2] This view was deduced from the findings that one of T1 and T2 was modified much more slowly than the other in Eo modification and that the modifiable one on all 1 : 1 complexes was modified rapidly. Thus, the Trp residue which participates in the binding of the first ligand molecule was arbitrarily designated as T1. This was substantiated here by the isolation of T1P and HNB-T2P from the above three 1 : 1 complexes.

In the previous study (Table I), we could reasonably assign the Trp residues involved in the binding, assuming that ligand binding protects the concerned Trp residue from modification, although with the exception of E-DDT_2 and E-DDT_9. The modification pattern of E-DIM_2 was explained in terms of protection from modification of a concerned residue by ligand binding. The modification pattern of E-DCBP and E-$DCBP_2$ was explained as the second molecule binding to the 1 : 1 complex at a site not involving a Trp residue.[2] On the other hand, the number of modifiable Trp residues of E-DDT_2 decreased excessively with increasing number of bound ligands in Ew modification when compared with E-DDT, and unexpectedly increased in Eo modification. In addition, in W modification of E-DDT_9, T6 and T7, which were not modified in E-DDT_2, became modifiable upon E-DDT_9 formation, supposedly by the exposure to T6 and T7. The exposure of T6 and T7 observed in E-DDT_9 and E-aldrin$_7$ was previously discussed in connection with their enhanced enzyme activity (4.4- and 16-fold, respectively) toward tripropionin.[2] These unexpected results with E-DDT_2 and E-DDT_9 were explained as arising from the change of environment around the Trp residues or conformational changes. The results in Table II directly support the previous explanation.

Fortunately, Trp- and HNB-Trp-containing peptides were small, and each had a different amino acid composition. They were isolated and purified in *ca.* 65% yield to over 98% purity with regard to amino acid composition. Trp-containing peptides were virtually not detected on HPLC when the corresponding HNB-Trp-containing peptides were obtained, and *vice versa*. This means that the modifications in the three different conditions were selective for the particular Trp residues and that the number of modified residues did not represent a consequence of incomplete modification of unspecified Trp residues. The present study also confirms the classification by Chiba *et al.*,[4] which was presented on the basis of the number of modified Trp residues in W, Ew and Eo modification, re-modification of the modified protein and the change of enzyme activity caused by modification.

Experimental

Materials TPCK-Trypsin was purchased from Sigma Chemical Co., and DABITC and dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide (DHNBS-Br) were from Wako Pure Chemical Industries, Ltd.

Modification of Trp Residues on Pesticide–Lipase Complexes Modification of the native lipase and pesticide-lipase complexes in the absence of 6 M urea were performed essentially the same as in the previous report[2] with the slight modification that, in the final step, gel filtration to separate the product from reagent was done using water as an eluent instead of buffer.

W modification in the presence of 6 M urea was carried out as follows. A powder of DHNBS-Br, in a molar ratio of 400 : 1 to protein, was added to a 0.1 M sodium acetate buffer (6 ml), pH 6.0, containing pesticide-lipase complex (0.1—0.2 μmol) and 6 M urea over 30 min, and the mixture was stirred continuously for 6 h. After removal of separated hydroxynitrobenzyl alcohol by centrifugation at 3000 rpm for 10 min, the supernatant was applied to a Sephadex G-25 column (1.2 × 50 cm) and the column was eluted with water. Modified pesticide-lipase complex migrated as a fast-moving yellow band and was pooled in *ca.* 10 ml of eluate practically free of salt and urea.

Isolation of Trp-Containing Peptides Proteins and HNB-proteins were reduced and S-carboxymethylated according to Crestfield *et al.*,[7] and the RCM-proteins were separated from excess reagents by gel filtration. The RCM-proteins were dissolved in 0.2 M NH_4HCO_3 (pH 7.8) and digested with TPCK-trypsin (at a trypsin/substrate ratio of 1 : 20) at 37 °C for 24 h. The digest was lyophilized and dissolved in 20% CH_3CN containing 0.05% trifluoroacetic acid (TFA). The insoluble material contained the majority of T1P or HNB-T1P. The solution was chromatographed on a TSKgel ODS-80TM column (7.8 × 300 mm, Tosoh) with a linear gradient from 20% to 56% CH_3CN containing 0.05% TFA for 40 min at a flow rate of 5.0 ml per min using a Tosoh HPLC system. Elution of peptides was detected by recording the absorbance at 280 nm. Each fraction containing peptide was monitored by measuring absorbance at 410 nm (ε, $1.80 \times 10^4\ M^{-1}\ cm^{-1}$) in 0.1 N NaOH for HNB-Trp residue[4] and by fluorometric measurement for Trp residue. The fractions containing Trp- or HNB-Trp-containing peptide were further chromatographed on a TSKgel Octadecyl-4PW column (4.6 × 75 mm, Tosoh) with a linear gradient from 20% to 48% CH_3CN containing 150 mM NH_3 for 20 min at a flow rate of 1.0 ml per min. Further purification was carried out by rechromatography.

The material insoluble in acidic 20% CH_3CN was dissolved in 45% CH_3CN containing 120 mM NH_3 and purified by chromatography on an Asahipak C4P-50 column (4.6 × 250 mm, Asahi Chemical Industry Co., Ltd.) with a linear gradient from 34 to 70% CH_3CN containing 120 mM NH_3 to give purified T1P or HNB-T1P.

Sequence Analysis Manual sequence analysis of peptides was carried out by the liquid-phase DABITC method.[5] The released 4-*N*,*N*-dimethylaminoazobenzene-4′-thiohydantoin amino acids were analyzed using a TSKgel ODS-80TM column (4.6 × 250 mm, Tosoh).

Amino Acid Composition of Trp-Containing Peptides Peptides (100—300 μg) were hydrolyzed in 100 μl of 6 N HCl containing 4% thioglycolic acid at 110 °C for 22 h and analyzed using an amino acid analyzer (L8500, Hitachi). HNB-Trp was not determined by the analyzer, but by measuring the absorbance at 410 nm of the HNB-peptides in 0.1 N NaOH.

The yields of peptides in Table II were calculated from amino acid analyses on the basis of protein used. Counterpart peptides (peptide or HNB-peptide) were less than 2%, though T6P was found at a yield of *ca.* 4% of HNB-T6P in Ew modification of native lipase. Contaminative amino acid was less than 2% and data on amino acid analyses were omitted.

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