The Chemical Modification of the Lipase of *Humicola lanuginosa* by N-Bromosuccinimide in Urea Solution†

Wen-Hsiung LIU,* Teruhiko BEPPU and Kei ARIMA

Department of Agricultural Chemistry, The University of Tokyo, Tokyo

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The chemical modifications of the lipase of *Humicola lanuginosa* by various reagents were investigated in the presence of urea. It was found that the lipase was completely inactivated by the addition of N-bromosuccinimide (NBS) in both the presence or absence of urea. According to the change of UV-spectra at 280 nm and 260 nm, it was confirmed that tryptophan residues of the *Humicola* lipase were specifically oxidized by N-bromourea which was produced by the reaction of NBS with urea. The lipase activity disappeared completely along with the oxidation of two of the three tryptophan residues in the lipase molecule.

The chemical modification method has been widely used by many investigators to identify which amino acid residues are functioning intimately in the catalytic activity. It is well known that lipase (EC 3.1.1.3) has the unusual property of being functional only at an oil/water interface. It was postulated²⁾ that the catalytic center of lipase is formed and becomes able to interact with substrates and inhibitors only when the structure of enzyme molecule is slightly modified in an oil/water heterogeneous system by interfacial adsorption. Therefore, the chemical modifications of lipase have been generally carried out in an emulsified or micellar condition.^{3,4)}

In the previous report,¹⁾ it has been described that in the presence of 8 M urea, the α -helical structure of the *Humicola* lipase changes but the full activity still retained after urea is removed. Thus, this property is very favorable for making an attempt to perform the chemical modification of the enzyme in a urea solution instead of oil/water heterogeneous system, which makes inner residues of the protein molecule reactive. On the other hand, modification of the proteins by N-

bromosuccinimide (NBS) in urea solution is very useful, since N-bromourea (NBU) produced from NBS and urea behaves as a very specific oxidizing agent of tryptophan residues.⁵⁾ The present report is concerned with the chemical modification of the *Humicola* lipase by NBS in urea solution and the role of tryptophan residues on the lipase activity.

MATERIALS AND METHODS

Preparation of enzyme. The enzyme used in this study was prepared as described previously⁶⁾ and confirmed to be homogeneous with disc electrophoresis and ultracentrifugal analyses.

Estimation of lipase activity. Lipase activity was estimated by the olive oil emulsion method as described previously.⁷⁾ One unit of lipase activity was defined as the amount which liberated 1 μ mole of free fatty acid per minute at 45°C and pH 8.0.

Oxidation of enzyme by N-bromosuccinimide. The oxidation of the *Humicola* lipase was performed in a 1.0 cm (4.0 ml) cuvette at room temperature. To 3 ml of the enzyme solution (dissolved in 0.1 M McIlvaine buffer pH 5.0, 5.5×10^{-5} M), 1 ml of 1×10^{-2} M N-bromosuccinimide (NBS) which dissolved in 0.1 M McIlvaine buffer (pH 5.0) with or without 16 M urea was added with continuous stirring. N-Bromourea (NBU) was produced when urea was present. The spectrum was scanned from 340 nm to 240 nm in various reaction times with a Hitachi recording spectrophotometer. The extent of tryptophan oxidation was estimated by the Spande's method.⁸⁾ The general equation is: % oxidation = $[(\Delta O.D./O.D.)100]$ ($1.31 \times \text{mol}$.

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* Present address: Laboratory of Fermentation, Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, Republic of China.

wt./1000 $\times$ V/5.50 f nV), where Δ O.D.=corrected decrease in optical density at 280 nm; O.D.=initial optical density at 280 nm; 1.31=empirical factor; 5.50=millimolar extinction coefficient for tryptophan at 280 nm; mol. wt.=molecular weight of protein; V=sample volume, ml; f =optical factor for protein at 280 nm; n =number of tryptophan residues/mole of protein. The value of the *Humicola* lipase used to estimate the extent of tryptophan oxidation were as follows: molecular weight=27,500, n =3, and f =0.85.^{1,6)}

Chemicals. Chemicals with a reagent grade were obtained from Tokyo Kasei Kogyo., Ltd., and Kokusan Chemical Work, Ltd.

RESULTS

Effect of various chemical reagents on lipase activity

A number of chemical reagents were used to examine the influence of specific chemical modification on the activity of the *Humicola* lipase. Experiments were carried out at room temperature in a buffer system with or without urea. As shown in Table I, 2-mercaptoethanol,

TABLE I. EFFECT OF VARIOUS CHEMICAL REAGENTS ON LIPASE ACTIVITY

Chemical reagents	Final conc. (mM)	Relative activity (%) ^{a)}	
		Buffer system	Urea system
Control	—	100.0	100.0
N-Acetylimidazole	5.0	100.0	100.0
N-Bromosuccinimide ^{b)}	2.5	0.0	20.0
Teranitromethane	5.0	100.0	100.0
Diazonium-1-H-tetrazole	0.1	95.0	95.0
Monoiodoacetic acid	1.0	95.0	95.0
Diisopropylfluorophosphate	1.0	95.0	95.0
N-Ethylmaleimide	5.0	100.0	100.0
<i>p</i> -Chloromercuribenzoic acid	0.2	100.0	100.0
B-Naphthoquinone disulfonic acid	5.0	100.0	100.0
2-Mercaptoethanol	5.0	100.0	100.0
Dithiothreitol	5.0	100.0	100.0

^{a)} The mixtures which consisted of 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.0) with or without 16 M urea, 1 ml of various chemical reagents and 0.5 ml of 10^{-5} M lipase solution were incubated at 25°C for 30 min, then diluted with 0.05 M Tris-HCl buffer (pH 8.0) to 10 ml. The residual activity was determined as described in the text.

^{b)} 0.1 M McIlvaine buffer (pH 5.0) was used.

dithiothreitol, monoiodoacetate, N-ethylmaleimide and *p*-chloromercuribenzoate showed no effect on the lipase activity even in the presence of 16 M urea. It was indicated that the *Humicola* lipase did not require either free sulfhydryl group or an intact disulfide linkage for lipolytic activity. Diisopropylfluorophosphate and diazonium-1-H-tetrazole which are specific chemical reagents for the modification of serine and histidine residues, respectively, did not inhibit the lipase activity. On the other hand, the lipase was sensitively inactivated by addition of N-bromosuccinimide which was a specific chemical reagent for the oxidation of tryptophan and tyrosine residues of protein. As shown in Fig. 1, in the aqueous solution the enzyme activity vanished rapidly within a very short period as a consequence of NBS-treatment, while the enzyme activity decreased gradually by the action of the same reagent in the presence of urea.

FIG. 1. Effect of N-Bromosuccinimide on the *Humicola* Lipase Activity in the Presence or Absence of Urea.

$\times$, 2.5×10^{-3} M NBS without urea; $\bullet$, 2.5×10^{-3} M NBS with urea; $\circ$, 5.0×10^{-4} M NBS with urea.

The reaction conditions were the same as described in Table I.

Effect of various concentration of NBU solution on the lipase activity

The mixture of 0.5 ml of the lipase solution (5.5×10^{-5} M), 0.5 ml of various amounts of NBU solution and 1 ml of 0.1 M McIlvaine

FIG. 2. Effect of N-Bromosuccinimide Concentration on the *Humicola* Lipase Activity in the Presence of Urea.

Incubation time: ○, 30 min; ●, 60 min.

buffer (pH 5.0) were preincubated at 25°C for 30 min and 60 min. After dilution with 0.1 M McIlvaine buffer (pH 5.0) to 15 ml, the residual activities were determined and the results were shown in Fig. 2. It was found that the inhibition of the lipase increased as the amounts of NBU increased. In the presence of 2.5×10^{-3} M and 5.0×10^{-3} M NBU, the lipase was completely inactivated at room temperature within 60 min and 30 min, respectively.

Ultraviolet absorption spectra of the lipase preparations treated with NBU and NBS

The change of UV-spectra of the lipase due to the treatment with NBU or NBS was measured at various reaction times by a recording spectrophotometer. As shown in Fig. 3, they showed a small decrease at 280 nm by the treatment, indicating that the indole ring of tryptophan residues in the lipase were brominated by NBU. At the same time, they showed more distinct increase at 260 nm due to an intensification and red shift of the oxyindole absorption band as a result of bromination. The decrease at 280 nm and increase at 260 nm due to the bromination of indole ring of tryptophan residues by NBU were quite obvious. On the other hand, the change of UV-spectra of the lipase due to the NBS-treatment

FIG. 3. Ultraviolet Absorption Spectra of the *Humicola* Lipase Treated with N-Bromosuccinimide in the Presence of Urea.

Incubation condition: pH 5.0, 25°C. Curve A, 0 min; B, 2 min; C, 10 min; D, 30 min; E, 60 min.

FIG. 4. Ultraviolet Absorption Spectra of the *Humicola* Lipase Treated with N-Bromosuccinimide in the Absence of Urea.

Incubation condition: pH 5.0, 25°C. Curve A, 0 min; B, 2 min; C, 10 min; D, 30 min; E, 60 min.

occurred rapidly but was not so specific (Fig. 4). In this case, some other chemical reactions may also have occurred in addition to the bromination of tryptophan residues of the lipase molecule. From the decreased amount of optical density at 280 nm of the NBU-treated lipase, it was approximately calculated that the lipase was completely inactivated when about 55% of the tryptophan residues in the lipase molecules was oxidized (Fig. 5).

FIG. 5. Relation between Lipase Activity and Tryptophan Oxidation during the Course of N-Bromosuccinimide Treatment with Urea.

○, residual activity; ●, tryptophan oxidation; ×, decrease in O.D.₂₈₀; ▲, increase in O.D.₂₆₀.

DISCUSSION

Many attempts have been made to confirm whether sulfhydryl groups were involved in the catalytic center of lipases. But it is now generally agreed that lipase is not considered as a true sulfhydryl enzyme.²⁾ As shown in Table I, the *Humicola* lipase was not inhibited by treatment with various sulfhydryl reagents. It was apparent that sulfhydryl groups were not concerned with the lipolytic function of the *Humicola* lipase.

It was reported that histidine participated in the activity of pancreatic lipase, in conjunction with methionine⁹⁾ or serine.^{10,11)} In the present study, these results were not obtained by using specific chemical reagents (Table I). These amino acid residues seemed not to relate to the lipolytic function of the *Humicola* lipase.

N-Bromosuccinimide has been widely used

for cleavage of both tryptophanyl and tyrosyl peptide bonds in proteins and for the approximate estimation of tryptophan by spectrophotometric titration.¹²⁾ Under mild condition, however, the reagent selectively oxidized the tryptophan and tyrosine residues without significant cleavage of these peptide bonds as observed with trypsin and chymotrypsin.^{8,13)} Funatsu⁵⁾ has reported that in the urea solution, N-bromosuccinimide reacts with urea to form N-bromourea which then replaces NBS as an oxidizing agent. N-Bromourea reacts with tyrosine residues at a rate 250 times more slowly than NBS do in aqueous system. It is suggested that the chemical modification of tryptophan residues in protein by NBS is advantageous to be carried out in urea solution, both to unfold the protein molecule and to reduce nonspecific modification of tyrosine residues.

It is reported⁴⁾ that tryptophan residues are involved in the lipolytic function of the *Rhizopus delemar* lipase. As shown in Fig. 3, tryptophan residues of the *Humicola* lipase is specifically oxidized by NBU. It is estimated that when about 55% of the tryptophan residues in the lipase molecule is oxidized, the lipase is completely inactivated (Fig. 5). In the previous paper,¹⁾ it has been described that the *Humicola* lipase contains three tryptophan residues per molecule. It is concluded that the lipase activity is lost completely along with the oxidation of two of the three tryptophan residues.

It is well known that tryptophan plays an important role in determining and stabilizing the tertiary structure of a protein by its interaction with other hydrophobic residues. Meanwhile, its hydrophobic interaction is very important in the binding of enzyme and substrate.^{14~16)} It has been described¹⁾ that the *Humicola* lipase possesses a certain flexibility of hydrophobic structure and 3 pairs of disulfide linkages playing an important role to maintain a compact enzyme structure. It is reasonable to consider that 3 tryptophan residues in the *Humicola* lipase molecule might play an important role in maintaining the

active conformation of the protein. However, the possibility that tryptophan residues may be involved more directly in the lipolytic function of the enzyme still can not be excluded. It is apparent that the hydrophobic interaction of tryptophan is favorable for the binding of lipase and substrate at an oil/water interface. However, it is not clear that the oxidation of tryptophan residues of the *Humicola* lipase resulted in which of the following alterations: the inactivation of the active site directly, interference of the active site sterically or the conformation change of the protein. To determine which one of these is the actual case, it requires still additional evidences concerning the function of tryptophan residues in the *Humicola* lipase.

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