ORIGINAL

Chemistry of Succinimido Esters. IX.*¹Chemical Modification of Soybean Lipoxygenase with *N*-Succinimidyl Benzoates and the Oxidation of Linoleic Acid in Hexane-Buffer Biphasic System

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Soybean lipoxygenase I (LG I : pH optimum of 9.0) was readily *N*-arylcacylated by *N*-succinimidyl benzoates (1) and considerably stable up to a high modification ratio. The rate of self-catalyzed destruction of the modified enzyme was less than that of the native one in a borate buffer (pH 9.0).

The modification caused expression of the enzyme activity for bringing about oxidation of linoleic acid in hexane-buffer (pH 6.8) biphasic system. The native enzyme showed no activity due to its instability under the experimental conditions. Activity and stability increased with the modification ratio. When bovine serum albumin (BSA) modified by (1) was added to this system, nonmodified LG showed the activity for causing oxidation. From the above results, it is concluded that imparting hydrophobicity to LG I itself and/or the reaction system is an important factor for expression of enzyme activity in a biphasic system.

1 Introduction

N-Succinimidyl ester of carboxylic acid is known to react specifically with amino residues in sphingolipid base^{1),2)} and amino acid ester³⁾ because of the activation of the ester carbonyl residue by electron-withdrawing nature of the *N*-succinimidyl residue. In the previous papers⁴⁾⁻⁶⁾ on the reactivity of *N*-succinimidyl benzoates (1) with amino compounds, we showed that (1) could be used for the *N*-arylcacylation reagent of amino compounds in aqueous solution and chemical modification reagent of Lys residue in protein.

Recently, it was also reported that modified (acylated) lipoxygenase (LG) with *N*-acyloxysuccinimides showed enzyme activity for the oxidation of linoleic acid in the hexane-water biphasic system^{7),8)}.

In this paper, we selected (1) as a modifier and studied the oxidation of linoleic acid in the hexane-water biphasic system to clarify the detail mechanism for the occurrence of the enzyme activity under these conditions. Furthermore, the kinetic constants of the modified enzyme in aqueous solution was also measured.

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2 Experimental

2.1 Materials

Soybean lipoxygenase (LG) was purchased from Serva Co., Ltd. and LG I (pH optimum of 9.0, LG I was simply signified by LG in the followings) was purified by chromatography on DEAE-TOYOPEARL eluted with a linear sodium phosphate buffer gradient (0.01~0.3 M) at pH 6.8 according to the procedure of Christopher *et al.*⁹⁾. Disc electrophoresis on polyacrylamide gel showed the enzyme as one distinct band.

N-Succinimidyl benzoates (1) were prepared from the coupling reaction of benzoic acids (GR grade, from Tokyo Kasei Kogyo Co., Ltd.) and *N,N'*-disuccinimidylcarbonate (used for the synthesis of peptides, from Wako Pure Chemicals) by the previously reported method¹⁰⁾. Sodium 2,4,6-trinitrobenzenesulfonate (TNBS : GR grade), sodium sulfite and linoleic acid (> 99 %, octadeca-*cis*-9-*cis*-12 dienoic acid), were

purchased from Nakarai Chemicals. Acetonitrile and hexane used were HPLC analytical grade of Wako Pure Chemicals and other solvents were purified by the conventional method¹¹⁾. Other chemicals were of reagent grade of commercial products.

2.2 Chemical modification

To a solution of LG dissolved in phosphate buffer (pH 7.5, 1 mg/mL, 10 mL) was added the solution of (1) dissolved in dioxane ($5 \times 10^{-4} \sim 10^{-2}$ M, 1 mL). The mixture was incubated at 25°C for 15 h, and these modified enzyme solutions were dialyzed against sufficient phosphate buffer (pH 6.8, 0.01 M) at 4°C. This solution was used for the subsequent experiments. Chemical modification of BSA by (1) was also carried out with the above method.

LG concentration was estimated from the absorbance at 280 nm assuming $E_{1\text{cm}}^{1\%} = 14.3^{12)}$. Modified LG concentration was measured with the method of Lowry¹³⁾ on the basis of the above value. Modification ratio were measured by the extinction at 420 nm of Meisenheimer complex using TNBS and Na_2SO_3 with the method reported previously¹⁴⁾.

2.3 LG reaction in aqueous solution

The oxygenation reactions were initiated by addition of aliquots of solutions of LG (2~30 μL , 1 mg/mL) to 3.0 mL of 0.05 M borate buffer (pH 9.0) containing freshly prepared sodium linoleate (50~150 μM). Assays of LG were performed at 25°C in a cuvette exposed to air by continuous monitoring the absorbance at 234 nm due to the formation of the hydroperoxy diene products on the JASCO UV-430 spectrophotometer. A molar extinction coefficient of 28,000 ($\text{M}^{-1} \cdot \text{cm}^{-1}$)¹⁵⁾ was used to convert absorbance readings to moles of diene products. Under these conditions, this enzyme reaction occurred with a kinetic lag period which might be due to the activation of the enzyme by the products¹²⁾. In order to remove the kinetic lag period, diene products ($>5 \mu\text{M}$) which was produced by LG were added to the substrate solution before initiating the reaction.

The rate constants for the inactivation (k_a) of LG during the reaction were measured by the method reported by Smith *et al*¹²⁾.

Michaelis constants (K_m) for LG catalyzed oxidation of sodium linoleate in the buffer solu-

tion were determined by the nonlinear least-squares method using Taylor expansion¹⁶⁾.

2.4 LG reaction in biphasic system

A mixture of buffer solution (pH 6.8, 0.05 M) containing LG solution (0.5 mg/mL, 2 mL) and hexane solution (25 mL) containing linoleic acid (2.5 mg/mL) was stirred at over 500 rpm at 30°C. After the appropriate time, an aliquot of a hexane solution was evaporated in an atmosphere of nitrogen and diluted with 10 % water-methanol containing 0.1 % phosphoric acid, and absorbance at 234 nm was measured. The residual activity of LG was measured at pH 9.0 as described in 2.3.

2.5 Analysis of the products

The reaction products in aqueous and biphasic systems were analyzed by the method reported previously^{7),8)}.

3 Results

3.1 Chemical modification of LG

Amino residues which were located on the surface of the LG could be *N*-arylcabonylated by (1) in the phosphate buffer (pH 7.5, 0.1 M) at 25°C.

Fig. -1 indicates the decrease of activity of LG modified by (1a) in a borate buffer (pH 9.0, 0.05 M). The enzyme activity did not almost change until the modification ratio of 70 % and decreased gradually over that. The same results were obtained for other reagents (1b)~(1d). No significant change in circular dichroism spectrum (200~250 nm) of LG by the modification was observed. These observations suggested that LG was stable against the modification by (1).

In the previous paper⁸⁾ on the modification with

Fig. -1 Chemical modification of LG by (1a). Plots of relative activity vs. the modification ratio.

N-acyloxysuccinimide, we reported that the decrease of LG activity modified with CN=2, 4, 6 (CN : carbon number of acyl residue) was small but it became large for CN=8. The phenomenon of the decrease of the LG activity by modification with (1) seems to correspond to that with CN=6.

3.2 Activity of the modified LG in the aqueous solution

3.2.1 Lag period (τ)

A kinetic lag period (τ) was reported for the LG reaction in the absence of the hydroperoxide products¹²⁾. The lag period for the modified LG was measured at pH 9.0 and 25°C. The results are summarized in Table-1, which shows the decrease in lag period (τ), that is, LG was activated by the modification with (1).

3.2.2 $V_{\max}$ and K_m

The representative Michaelis constant (K_m) and the maximum velocity ($V_{\max}$) were determined for the LG reaction in the presence of dienes (2.3) at 25°C. The representative Michaelis-Menten plots were shown in Fig. -2. The values of $V_{\max}$ and K_m were also listed in Table-1. $V_{\max}$ decreased slightly with increasing the modification ratio and was independent of the substituent in (1). But K_m values of sodium linoleate for modified LG were larger than that for

Table-1 The effects of the modification on lag period, $V_{\max}$, K_m and inactivation constant (k_d) for the oxidation of sodium linoleate in the borate buffer¹⁾.

(1)	Modification ratio (%)	Lag period (τ)(s)	$V_{\max}$ (μ M/s)	K_m (μ M)	$10^2 k_d$ (min^{-1})
(1a)	0	18.1	0.78	15.8	2.18
	50	10.0	0.76	30.7	1.80
	72	8.2	0.74	31.0	1.52
	82	9.3	0.70	30.9	1.45
	84	11.3	0.66	32.4	1.61
	91	10.8	0.60	34.2	1.74
(1b)	61	9.2	0.75	30.1	1.68
	80	10.1	0.68	32.1	1.55
(1c)	60	9.8	0.71	32.3	1.70
	85	11.2	0.62	33.0	1.60
(1d)	58	9.9	0.74	31.5	1.81
	77	11.3	0.69	34.8	1.53

a) pH 9.0 (0.05 M), $T=25^\circ\text{C}$, enzyme concentration : 1 $\mu\text{g/mL}$.

Fig. -2 Plots of $1/v$ vs. $1/s$ for the oxidation of sodium linoleate catalyzed by native LG (○) and modified LG by (1a) (●) (modification ratio : 91 %) in borate buffer (pH 9.0, 0.05 M) at, 25°C and enzyme concentration of 1 $\mu\text{g/mL}$.

native one. Previously, we reported that K_m values for LG modified by CN=2, 4 and CN=6, 8 were about 30 and 50 μM , respectively⁸⁾. The values of K_m for LG modified by (1) seems to correspond to those for CN=2, 4.

3.2.3 Self-catalyzed destruction

The active site of LG I was destroyed during the enzyme reaction catalyzed the oxidation of sodium linoleate in the borate buffer. The kinetic model for self-catalyzed destruction of LG was reported by Smith *et al*²⁾ as follows :

$$V_t = d[\text{P}]/dt = \frac{k_d[\text{P}]}{\frac{K_m}{[\text{S}]} + 1}$$

where E, E*, P and S are active and inactivated LG, product and substrate, respectively. Since the reaction was carried out under the conditions of $[\text{S}] > K_m$, the values of the first order rate constants (k_d) were determined from the slopes of the plots of instantaneous velocity versus substrate concentration at time t . The k_d values of modified LG were smaller than that of native LG as shown in Table-1. This result means that the stability of LG during the reaction increases by the modification.

3.3 LG reaction in the hexane-buffer biphasic system

3.3.1 Appearance of the enzyme activity in the biphasic system by the modification

Native LG (Non-modified LG) was unstable to hexane, and inactivated rapidly in the biphasic system, *i. e.*, only a small amounts of conjugated dienes were produced for the oxidation of linoleic acid. On the other hand, modified LG by (1) was stable for a certain period and accumulation of the diene products in hexane was observed (Figs. -3 and 4). The activity of modified LG in the biphasic system increased with increasing modification ratio. Similar results were obtained with respect to (1b) and (1d). These show that activity of the modified LG is almost independent

Fig. -3 Time course for the oxidation of linoleic acid catalyzed by LG modified with (1a) in the biphasic system at 30°C. Modification ratios were designated in parentheses.

Fig. -4 Time course for the oxidation of linoleic acid catalyzed by modified with (1c) in the biphasic system. Modification ratios were designated in parentheses.

of substituent in the modifier.

3.3.2 Stability of LG in the biphasic system

Stability of LG in the biphasic system in the absence of the substrate at 30°C was examined. The results are shown in Fig. -5. The non-modified LG was inactivated completely for about 50 min. On the other hand, the enzyme was stabilized by the modification with (1a) and the half life periods of the modified LG increased with increasing the modification ratio. In the presence of linoleic acid, the half life periods of modified LG were somewhat (20~30 %) longer than that in the absence of linoleic acid. Similar results were obtained with respect to (1b)~(1d).

Fig. -5 Stability of LG in the hexane-buffer biphasic system in the absence of linoleic acid at 30°C. Modified LG in buffer=0.5 mg/mL. Modification ratios were designated in parentheses.

3.3.3 Effect of the additives

The effect of the additives on the oxidation of linoleic acid catalyzed by native LG was examined. BSA, glycerol, casein and NaCl which are thought to be stabilizers of the enzymes and methanol to be the surface activator between hexane and water showed no effect on the reactivities. On the other hand, the addition of the modified BSA by (1a) (modification ratio=80 %) was found to activate native LG in the biphasic system, and the conjugated dienes were accumulated in the hexane layer as shown in Fig. -6. The activity increased with increasing of the concentration of modified BSA (Fig. -6).

3.3.4 Analyses of the products

In the biphasic system, the oxidized products were analyzed by HPLC. Three main products (13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic

Fig. -6 Effect of BSA modified by (1a) in the biphasic system. Time course of the conversion of linoleic acid catalyzed by native LG in the biphasic system at 30°C. Concentration of modified BSA was designated in parentheses.

acid, 13-hydroperoxy-*trans*-9-*trans*-11-octadecadienoic acid and 9-hydroperoxy-*cis*-10-*trans*-11-octadecadienoic acid) were detected. And these products and their ratios of these were the same as those for the biphasic reaction catalyzed by modified LG with *N*-acyloxysuccinimides.

4 Discussion

4.1 Improvement of LG functions in the aqueous solution by the modification

LG was stable against the modification with (1) and the activity was remained until the modification ratio of 70 % (Fig. -1). The lag period¹²⁾ of this enzyme reaction in the buffer solution was decreased by the modification (3.2). These results suggest that the modified LG was more activated by the product or less inhibited by the substrate (sodium linoleate) in the process of the activation of the enzyme. Furthermore, the first order rate constant (k_d) for the self-catalyzed destruction of LG decreased by the modification (Table-1), that is, the stability of LG during the reaction increased by the modification. K_m value of sodium linoleate was increased by this modification, that is, the affinity of the active center in LG for sodium linoleate decreased. The similar improvement of enzyme functions was also reported for the chemical modification by *N*-acyloxysuccinimides⁸⁾. From the above facts, it is speculated that the amino residues modified by (1) are not located at

the active center of LG, but somewhat related to the inactivation step ($[E \cdot O_2 \cdot P \cdot S] \rightarrow E^*$) and the activation step by the product.

4.2 Appearance of LG activity in the hexane-buffer biphasic system

Usually, LG activity is observed in the buffer solution containing sodium linoleate as substrate or containing linoleic acid dispersed with surface-active agent (Tween 20 *etc.*)⁹⁾. But we encountered difficulties of the reaction at the high substrate concentration and extraction of the products. Organic-aqueous biphasic system was able to eliminate them. In this system, the reaction seems to be carried out at the interface between hexane and water. Native LG was unstable in the presence of hexane and no reactivity was observed in the system. However, the modified LG endowed with hydrophobicity by (1) was somewhat stable and showed the activity. The stabilities and reactivities increased with increasing the hydrophobicity by the modification, but they were independent of the substituent in (1), *i. e.*, these phenomena were due to the aryl group in (1) not to the substituent groups of (1).

Addition of BSA modified by (1) also caused the enzyme activity of native LG in the biphasic system, although casein, BSA, glycerol, NaCl and methanol showed no positive effects. The activity increased with increasing the concentration of modified BSA. These results indicate that the *N*-arylcarbonylated parts of the proteins by (1) served as stabilizers and activators of this enzymes in the biphasic system. This demonstrates that in the case that enzymes are inactivated by this modification, additions of other proteins modified by (1) may stabilize and activate the enzymes in this system.

The activity of the modified LG by (1) is comparable with that by *N*-acyloxysuccinimides (CN=6 and 8). However, the activity in the presence of BSA modified with (1) was smaller than that with *N*-acyloxysuccinimides (CN=6 and 8). These conclude that additional hydrophobicity to LG itself and/or the biphasic system is an important factor for causing LG activity in it.

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スクシンイミドエステルの化学 (第9報)

N-スクシンイミジルベンゾエートによる大豆

リポキシゲナーゼの化学修飾及びヘキサン- 緩衝液二相系でのリノール酸の酸化反応

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二相系(水-有機溶媒)において酵素反応を行うことを目的とした。モデル酵素として、ダイズより得られたリポキシゲナーゼ(LG)を用い、*N*-スクシンイミジルベンゾエート(1)でアミノ基を修飾し二相系(水-ヘキサン)での酵素反応を試みた。

基質(リノール酸)を溶かしたヘキサン溶液に、酵素を溶かした水溶液を加えた二相系においては、未修飾のLGは、不安定でヘキサン中のリノール酸はほとんど酸化されなかった。しかし、(1)で修飾したLGはある程度ヘキサンに対し安定であり、またヘキサン中のリノール酸の酸化が観察された。また安定性及び反応性は、修飾率が高い程大きく、すなわち酵素の疎水性を上げる程安定性及び反応性は、増大することがわかった。また、このような二相系での反応性は(1)で修飾したBSAを加えることによっても見られるということから、(1)により修飾されたタンパク質が、この系での安定性及び反応性に寄与するものと考えられた。

以上の結果から(1)が酵素のヘキサンに対する安定性を高める修飾試薬として使用できるものと推論できる。