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Lipase Activity for Lipid Hydroperoxides

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Catalytic activity of lipase P (Amano) from *Pseudomonas fluorescens* for lipid hydroperoxides were examined, and in organic solvent the enzyme was found to catalyze acylation reaction of the hydroperoxy group in a stereoselective manner producing optically active hydroperoxy compounds, whereas in aqueous medium the lipase-catalyzed ester hydrolysis afforded a complicated mixture of unknown reaction products.

Key words : lipase, lipid hydroperoxide, hydroperoxide, *Pseudomonas fluorescens*

Introduction

A typical lipid enzyme, lipases catalyze chemical transformations of triglycerides, fatty acids and phospholipids, and their stereospecificity as well as catalytic activity are known to differ largely being dependent on their origins such as microorganisms, plants, animals, chemical structure of the substrates and also the reaction conditions including solvents and additives. The salient characteristics of most lipases are their broad substrate specificity and stability in organic solvents. Because of these useful characteristics, a number of lipases have been applied to optical resolutions of many prochiral and racemic substrates, including unnatural ones[1]. Lipid substrates for lipases very often have unsaturated fatty acyl groups in their molecules and these non-conjugate all-*cis* olefine structures are extreemly vulnerable to highly oxidative conditions provided by active oxygen species such as superoxide anion, hydroxy radical, hydroperoxy radical, alkoxy radical, carbon radical and some radical initiating metal ions. As a result, autoxidation of the unsaturated lipids easily occur affording hydroperoxides and decompositon products. In nature, many lipases may inevitably be exposed to these oxydized lipids and it is of importance to see whether such lipases can afford to utilize them as substrates or not. This report describes the action of lipase P(Amano) as a typical enzyme from *Pseudomonas fluorescens* on fatty acid hydroperoxides as well as on a hydroperoxide of triglyceride.

Result and Discussion

When hydroperoxy groups are introduced into fatty acids or any other compounds and are submitted to lipase-catalyzed reactions, it should be noted that the hydroperoxy group itself becomes a good nucleophile and may be involved in the catalytic process in the active site of the lipases since the hydroxy group as a nucleophile is also a major reactive group in the site. Therefore, if we conduct a lipase-catalyzed acylation of some alcohol having a hydroperoxy group in the molecule, the group may also participate in the reaction affording an acylated hydroperoxide, or the presence of the group may prevent the reaction by possible inactivation of the enzyme. This was our first concern and the following experiments were conducted using racemic 2-hydroperoxy-2-phenylethane (**1**) as a substrate in organic solvent (reaction **A**). Isopropenyl acetate was

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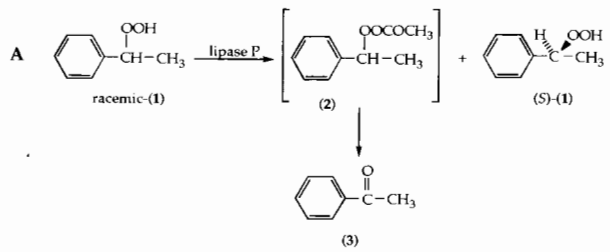


Fig. 1 Lipase-catalyzed optical resolution of 1-phenylethy hydroperoxide.

used as a lipase-catalyzed irreversible acylation reagent. Thus, a lipoprotein lipase, Amano P (2.0 g) was added to a solution of racemic **1** (4.1 mmol) and isopropenyl acetate (3.5 mmol) in cyclohexane (20 ml) and the solution was stirred at 25°C for 32 hr. After filtration of the enzyme, the filtrate was concentrated and the conversion ratio (49 %) of the hydroperoxide was measured by ^{1}H NMR. It is known that primary and secondary hydroperoxides are converted immediately to their carbonyl forms when the hydroperoxy groups are acylated. Therefore, in the present case too, if the lipase-catalyzed acylation occurs at the hydroperoxy group of the substrate **1**, acetophenone should be formed. In fact, a TLC analysis indicated formation of the keto compound. This suggests that the lipase retains the catalytic activity toward the hydroperoxy functional group, too.

This outcome intrigued us into conducting a similar experiment in which a racemic hydroperoxide (**4**) was examined as a substrate for the lipase-catalyzed acetylation under the same conditons (reactin **B**). As described in detail in the experimental section, the keto-form (**7**) was in fact obtained and the remaining hydroperoxide (**5**) unreacted was found to show optical activity of $[\alpha]_D^{rt}+6.5°$ (chloroform), (75 % *e.e.*). The two examples (reactions **A** and **B**) unambiguously indicated that the lipase can utilize the hydroperoxide substrate without apparent inactivation of the enzyme concerning with as far as the acylation reaction and in organic medium.

As a next step, we examined lipase-catalyzed

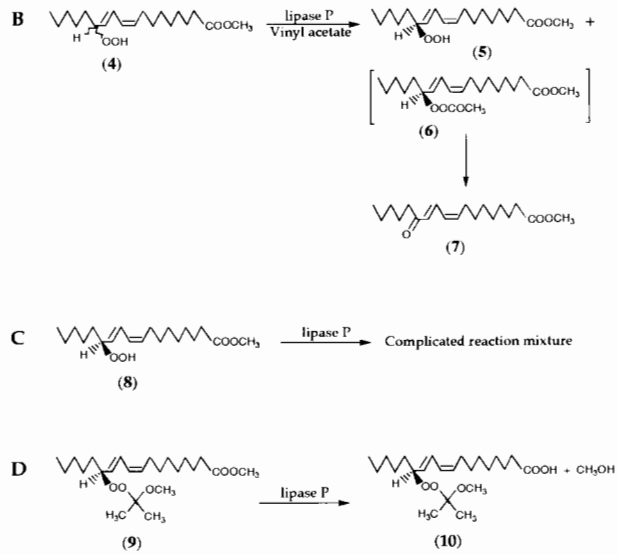
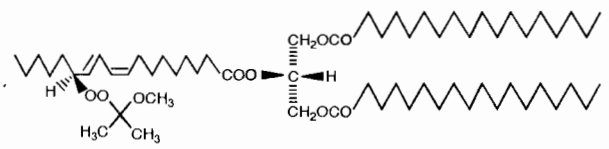


Fig. 2 Lipase-catalyzed optical resolution of racemic methy 13-hydroperoxyoctadecadienoate (reaction **B**), and lipase-catalyzed hydrolysis of methy 13-hydroperoxyoctadecadienoate (reaction **C**) and its peracetal (reaction **D**).

hydrolysis of ester bond in hydroperoxy fatty acid methyl ester. As a typical example, the same lipase was applied to **8** in a mixture of water and cyclohexane (1 : 1) at 30°C for 1 h (reaction **C**). Silica gel TLC of the reaction mixture, however, showed many spots and it was unclear whether the desired hydrolytic reaction occured or not. Interestingly, another substrate (**9**) in which the hydroperoxy group was protected as a peracetal was found to be cleanly hydrolyzed by the enzyme under the same conditions (reaction **D**). Although it is not clear whether the hydroperoxy substrate was decomposed to afford various compounds under the aqueous conditions by lipase-catalysis or whether the enzyme itself was decomposed by the hydroperoxide, the lipase P was found to show normal catalytic function toward hydroperoxy groups at least, in organic media, whereas in aquatic conditions, some unknown reactions appeared to occur.

In due course, we examined lipase-activity toward a triglyceride hydroperoxide (**12**), which is a close substitution for those formed by autoxidation of triglycerides having unsaturated fatty



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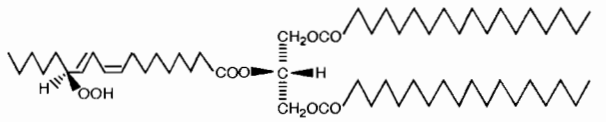

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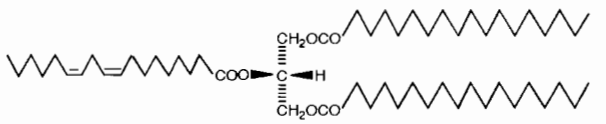

(13)

Fig. 3 Chemical structure of triglyceride peroxide (11), triglyceride hydroperoxide (12) and 1,3-distearoyl-2-oleoylglycerol (13).

acyl groups. The lipase-catalyzed hydrolysis was conducted under the same conditions as with **8**, and the stearic acid liberated from 1-and 3-position of the glyceride was determined by VPC, as described in the experimental section. It was found that the yield of the stearic acid liberated was 23 %, calculated from the moles of the substrate. By the same experiment for the other substrates, (**11**) and (**13**), the yields were 65 and 45 %, respectively. In these studies, since the stearoyl group to be cleaved by the enzyme does not carry a hydroperoxy function, we can not conclude explicitly that the differences in the chemical yields are due to the presence of OOH in the acyl group at 2-position. The lowest yield with **12** implicates some participation by the OOH group in the reaction. Yamane et al reported that autoxidized triglyceride prevented full activity of lipases for the hydrolysis and also the author found that the glyceride caused polymerization of the lipase protein[2].

As a conclusion, the present study showed that in organic medium the lipase showed almost full activity toward the hydroperoxy group in substrates which provided a strongly oxidative environment for the enzyme, whereas in aqueous solution the activity was not fully used and some unknown reactions seemed to occur.

Experimental Section

Lipase-catalyzed Acetylation of racemic 2-Hydroperoxy-2-phenylethane (**1**).

To a solution of **1** (4.5 mmol) and isopropenyl acetate (3.1 mmol) in cyclohexane (20 ml) was added lipase P (Amano) (2.0 g) and the mixture was stirred at 25°C for 32 h. After filtration of the enzyme, the filtrate was concentrated and the conversion ratio (49 %) of the hydroperoxide was determined by ^{1}H NMR. The filtrate, after concentration, was chromatographed on silica gel (hexane/ethyl acetate, 90 : 10), and acetophenone (**3**) was recovered as well as unchanged hydroperoxide. The optical rotation of the hydroperoxide (*S*)-(**1**) was measured {$[\alpha]_D^{25}$-75.6° (c=2.10, ethanol)}which was reduced to 1-phenylethanol with lithium aluminum hydride[3]. After chromatographic purification (silica gel, hexane/ethyl acetate, 90 : 10), the enantiomeric excess of the alcohol was calculated from the optical rotation. {$[\alpha]_D^{25}$-37.8° (c=2.93, chloroform), 70 % *e.e.* with excess of *S*-enantiomer}[4]. HPLC analysis {Chiralcel OB, JASCO Co., Ltd. hexane/2-propanol (9 : 1)}showed that the alcohol had 71 % *e.e.*, which was in accord with the above result. Based on this value, the maximum rotation of 1 was calculated as $[\alpha]_D^{25}$-107° in ethanol.

Preparation of Racemic Methyl 13'-hydroperoxy-(9'Z, 11'E)-octadecadienoate (**4**).

This compound was prepared by Corey's method[5]. IR ν_{max} (liquid film) cm^{-1} : 1674 (C=O), 3400 (OOH). ^{1}H NMR ($CDCl_3$, 500 MHz) δ : 0.88 (3H, t, J=6.6 Hz, CH_3), 1.2-1.7 (18H, m, CH_2 x 9), 2.3 (2H, t, J=7.2 Hz, CH_2COO), 3.66 (3H, s, OCH_3), 4.37 (ddd, 1H, J=8, 15 Hz, 13-H), 5.46 (dd, 1H, J=7.7, 13.8, 9-H), 5.56 (1H, dd, J=8, 15 Hz, 12-H), 6.00 (1H, t, J=11 Hz, 10-H), 6.57 (1H, dd, J=11, 15

Hz, 11-H), 7.90 (1H, dd, OOH).

Lipase-catalyzed Acetylation of Recemic Methyl 13-hydroperoxy-(9Z, 11E)-octadecadienoate (**4**).

Racemic hydroperoxide (**4**) (0.20 g, 0.6 mmol) was taken into a 5 ml measuring flask and filled up to 5.0 ml with vinyl acetate. Lipase P (Amano) (0.15 g) was added to the solution, and the mixture stirred at room temperature. The reaction progress was followed by a chromatoscanner at 250 nm and quenched at a conversion yield of 50 -75 % by filtering out the enzyme. The hydroperoxide (**5**) left in the solution without acetylation was isolated pure by silica gel column chromatography (hexane/ethyl acetate, 85 : 15), and the optical rotation was measured for this compound, from which % *e.e.* was calculated by using the reported maximum rotation $\{[\alpha]_D+9.1^\circ$ (chloroform)$\}$[6]. The ketone (**7**) was isolated from the corresponding column fraction, IR ν_{max} (liquid film) cm^{-1} : 1674 (C=O), 1745 (O-C=O). ^1H NMR ($CDCl_3$, 500 MHz) δ : 0.89 (3H, t, J=6.9 Hz, CH_3), 1.2-1.5 (16H, m, CH_2), 2.29 (2H, t, J=7.7 Hz, CH_2COO), 3.66 (3H, s, OCH_3), 5.6-6.0 (3H, m, =CH-CH=CH-), 3.66 (3H, s, OCH_3), 6.53 (1H, d, J=12 Hz, COCH).

Synthesis of 1,3-Distearoyl-2-[13'-(S)-hydroperoxy-(9'Z, 11'E)-octadecadienoyl]-sn-glycerol (**12**).

This compound was synthesized according to our previous report[7].

Hydrolysis of Methyl 13-(S)-(1'-methoxy-1'-methylethylperoxy)-(9Z, 11E)-octadecadienoate (**8**) *and Methyl 13-(S)-hydroperoxy-(9Z, 11E)-octadecadienoate* (**9**) *Catalyzed by Lipase P (Amano).*

A solution of the methyl ester (2.85×10^{-4} mol) in cyclohexane (2 ml) mixed with an aqueous solution of lipase P (Amano) (50 mg/2 ml) was stirred in the presence of a small amount of butylated hydroxytoluene in a nitrogen atmosphere. After the reaction, deionized water (20 ml) was added and the product was extracted with dichloromethane (3 times) and hexane (3 times). After washing with water, the solution was dried over anhydrous sodium sulfate. After concentration, to the residues was added a solution (1.0 ml) of diphenylbenzene (2.02 mg/ml) in 2-propanol for the former and a solution (1.0 ml) of 1-naphthylethanol (2.15 mg/ml) in 2-propanol for the latter as internal standards. 2-Propanol was added for each to a final volume of 25 ml and the solutions were analyzed by silica gel TLC (hexane/ethyl acetate, 85 : 15) for the former and 7 : 3 for the latter. The spot intensity was determined by a densitomer (a flying spot scanner). For the sample analyses, a calibration curve for each methyl ester was made by the same procedure.

Hydrolysis of (**13**) *Catalyzed by Lipase P (Amano).*

A solution of the hydroperoxytriglyceride (**13**) (5.45×10^{-5} mol), lipase P (Amano) (30 mg) and butylated hydroxytoluene (5-6 mg, an antioxidant) in phosphate buffer (5 ml, pH. 7.0, 10 mM) was stirred at 35°C for 2 h. After this period, excess diazomethane in ether was added to convert stearic acid liberated from the triglyceride to a methyl ester, and methyl palmitate (10.0 mg) was added to the solution as an internal standard. The final solution was analyzed by gas chromatography under general conditions for analyses of fatty acid methyl esters.

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過酸化脂質に対するリパーゼの触媒活性

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自然界において通常の環境でその役割を果たす酵素の多くは特殊な環境下においては活性を示さないか失活することが多い．不飽和脂肪酸を結合するトリグリセリドは食品等の劣化にともなって容易に自動酸化をうけて脂質過酸化物を生じ，ヒドロペルオキシドの高い反応性に加えてこれらは周囲に強い酸化的環境を生み出す．本研究ではこのような状況下に基質としての過酸化脂質に対してリパーゼがどのような活性を示すかを検討した．その結果，*Pseudomonas fluorescens* 由来のリパーゼP (Amano) はヒドロペルオキシドのアシル化反応において有機溶媒中で通常と変わらない触媒作用を示し，しかもその反応は立体選択的に進行することが明らかとなった．有機溶媒中，過酸化物の存在という特殊な環境下における上記生体触媒反応は光学活性過酸化物の生産の新しい方法としての可能性を示すものである．一方，水系では過酸化脂質に対して上記リパーゼは正常な触媒作用を示さずリノール酸より合成されたラセミ型過酸化不飽和脂肪酸エステルの過水分解に対して生成物の複雑な混合物を与えた．以上のように特殊な環境下における微生物由来のリパーゼの活性は反応条件の違いにより変化することが本研究で明らかとなった．