

Sterol Ester Hydrolytic Activity of Lipoprotein Lipase from *Pseudomonas fluorescens*¹⁾

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The activity of sterol ester hydrolase was found in the crude enzyme preparation of lipoprotein lipase from *Pseudomonas fluorescens*. The enzyme was purified by ammonium sulfate fractionation and chromatographies on diethylaminoethyl (DEAE)-cellulose, Sephadex G-75 and carboxymethyl (CM)-cellulose. The lipolytic activity and sterol ester hydrolytic activity at various stages in the purification were found in the same fraction and the ratio of both activities was constant.

The purified enzyme was found to be homogeneous by disc electrophoresis and SDS-polyacrylamide gel electrophoresis. Furthermore, one single protein peak which contained lipolytic and sterol ester hydrolytic activities was observed by isoelectric focusing. Therefore, it was concluded that the actions of lipoprotein lipase and sterol ester hydrolase will belong to a same enzyme protein.

The action of the purified enzyme for the cholesterol esters in human plasma was investigated. Cholesterol ester was hydrolyzed completely and the rate of hydrolysis of esters of long chain fatty acids was more rapid than that of short.

Lipoprotein lipase (glycerol ester hydrolase, EC 3.1.1.3) will catalyze the liberation of fatty acid from triglyceride in the form of chylomicrons or serum lipoproteins and they are widely distributed in various animal tissues and participate on the absorption and utilization of fat.³⁾ The enzyme has also been found in the culture medium of microorganisms, *Mucor* and *Pseudomonas* etc., and purified by Saiki, *et al.*⁴⁾ There are several lipases which have hydrolytic activity for water soluble esters⁵⁾ or phospholipid.⁶⁾ We found the sterol ester hydrolytic activity in the crude enzyme preparation of lipoprotein lipase from *Pseudomonas fluorescens*. In the present paper, it was observed that the relationship between the lipoprotein lipase and sterol ester hydrolase.

Methods and Materials

Assay of Lipolytic Activity—The reaction mixture contained 0.5 ml of 1% sesame oil emulsion (Fatgen; Dainippon Pharm. Co.) in 0.1 M phosphate buffer (pH 7.3) and 0.2 ml of 10% bovine serum albumin. The enzymic reaction was carried out by adding 0.3 ml of enzyme solution at 37° for 20 min and the reaction was terminated by adding 3 ml of a mixture of isopropanol, heptane and 2 N H₂SO₄ (40:10:1, by v/v). After adding 2 ml of heptane and 1 ml of water, the mixture was stirred vigorously and standing for 30 min. The free fatty acid extracted in 0.1 ml of the upper layer was assayed by the method of Maehata.⁷⁾ One unit of lipoprotein lipase was defined as the amount of enzyme which liberate 1 μmole of free fatty acid per min.

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Assay of Sterol Ester Hydrolytic Activity—The enzymic reaction was carried out by 0.5 ml of human plasma as a substrate and 0.1 ml of enzyme solution at 37° for 20 min. After the reaction was stopped with 3 ml of a mixture of ethanol and ethyl ether (3:1, by v/v), 1 ml of 1% digitonin in 60% ethanol was added and the mixture was kept at 5° overnight. The produced precipitate was removed by centrifugation and 1 ml of the supernatant was dried and the cholesterol ester was assayed by the modified Zak and Henly method.⁸⁾ One unit of sterol ester hydrolase was defined as the amount of enzyme which liberate 1 μ mole of free cholesterol per min.

Assay of Protein—The protein content during the purification was determined by the method of Lowry, *et al.*⁹⁾ and by ultraviolet absorption at 280 nm.

Disc Electrophoresis—Disc electrophoresis was carried out in accordance with the method of Davis¹⁰⁾ using 7.5% polyacrylamide gel at pH 9.4.

Determination of Molecular Weight—Molecular weight of the enzyme protein was determined by the method of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using 10% gel.¹¹⁾ Electrophoresis was carried out at a constant current of 8 mA/column for 3.5 hr.

Isoelectric Focusing—Isoelectric focusing was carried out in accordance with the method of Vesterberg and Svensson.¹²⁾ After the electrophoresis, enzyme activities, protein content and pH of the effluent from the column was assayed.

Thin-Layer Chromatography—Thin-layer chromatography with Wako G (Wako Pharmaceutical Co.) was used for separation of cholesterol esters in human plasma from the other lipids. The thin-layer was developed with a mixture of petroleum ether, ethyl ether and acetic acid (90:10:1, by v/v). Lipids were detected by spraying the phosphomolybdic acid.

Gas-Liquid Chromatography—Gas-liquid chromatography was used for the assay of the fatty acids in cholesterol esters of human plasma. The cholesterol esters obtained by thin-layer chromatography were methylated according to the method of Stoffel, *et al.*¹³⁾ The methyl ester was assayed by a gas chromatograph (GC-550F, Yanagimoto Co. Japan) with a stainless steel column (4 \times 2250 mm) packed by 3% ethylene glycol succinate on Chromosorb W.

Materials—Diethylaminoethyl (DEAE)- and caroxymethyl (CM)-cellulose were the products of Brown and Sephadex G-75 was obtained from Pharmacia, Uppsala, Sweden. All of the chemicals used in this study were commercial products.

Results

Purification of Lipoprotein Lipase

The crude enzyme preparation which was obtained with fractional precipitation by ethanol (65–85%) from the cultural broth of *Pseudomonas fluorescens* was used as starting material for the purification. One hundred grams of the crude enzyme were suspended in 1000 ml of 10 mM phosphate buffer (pH 7.0) and were constantly stirred for 3 hr. After the suspension was centrifuged at 7500 $\times g$ for 20 min, solid ammonium sulfate was added until 35% saturation. The solution was left for 3 hr under stirring and the precipitate which contained most of the lipolytic and sterol ester hydrolytic activities was collected by centrifugation. The precipitate was dissolved in 200 ml of distilled water and was dialyzed overnight against 10 mM acetate buffer at pH 5.0. After centrifugation, the enzyme solution was applied onto a DEAE-cellulose column (4 \times 60 cm) which was previously equilibrated with 10 mM acetate buffer at pH 5.0. Elution was carried out with the same buffer and the passed through solution which contained most of the enzymic activities was collected. The enzyme was concentrated in 80 ml by ammonium sulfate precipitation and was dialyzed against distilled water overnight. The enzyme solution was applied on the column (5.5 \times 110 cm) of Sephadex G-75 which was previously equilibrated with distilled water. The elution was carried out with distilled water and the chromatogram was shown in Fig. 1.

Fractions containing both lipolytic and sterol ester hydrolytic activities were dialyzed against 1 mM acetate buffer (pH 4.4) overnight. Then the dialyzed enzyme solution was

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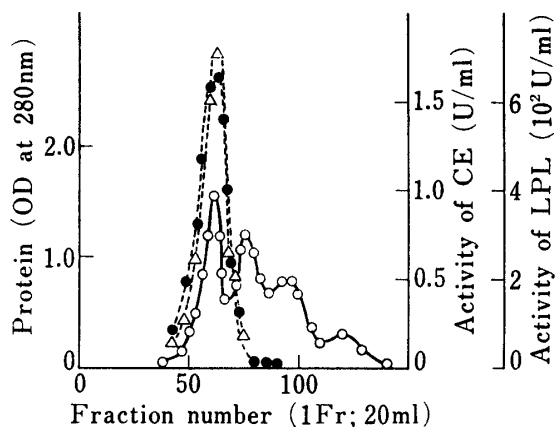


Fig. 1. Chromatography of the Lipoprotein Lipase from *Pseudomonas fluorescence* on Sephadex G-75

A column (5.5 × 110 cm) of Sephadex G-75 had been previously equilibrated with distilled water. The enzyme solution was applied onto the column and the elution was carried with distilled water at a constant flow rate of 40 ml/hr. The protein (○), activity of lipoprotein lipase (LPL; ●) and sterol ester hydrolase (CE; △) were assayed.

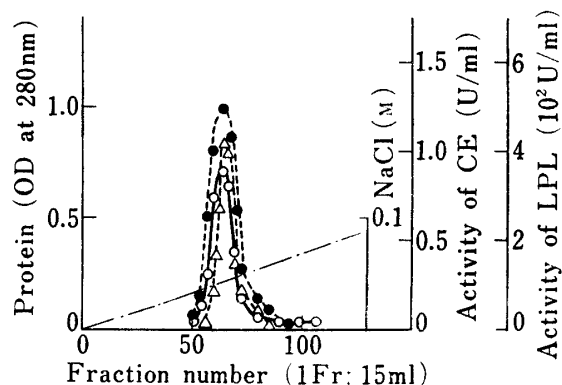


Fig. 2. Chromatography of the Lipoprotein Lipase on CM-cellulose

The enzyme solution was applied onto the column (3.5 × 60 cm) of CM-cellulose which was previously equilibrated with 1 mM acetate buffer at pH 4.4. Elution was carried out with a linear gradient from 1 mM acetate buffer (pH 4.4) to 1 mM acetate buffer (pH 5.5) containing 0.1M NaCl at a constant flow rate of 50 ml/hr. The protein (○), activity of lipoprotein lipase (LPL; ●) and activity of sterol ester hydrolase (CE; △) was assayed.

TABLE I. Purification of Lipoprotein Lipase from *Pseudomonas fluorescence*

	Lipoprotein lipase			Sterol ester hydrolase		
	Total activity (10 ⁵ U)	Specific activity (U/mg protein)	Yield (%)	Total activity (10 ³ U)	Specific activity (U/mg protein)	Yield (%)
Crude extract	2.96	2.70	100	0.649	0.006	100
Ammonium sulfate fraction	2.63	40.2	89	0.482	0.073	74
DEAE-cellulose elute	1.52	188.7	52	0.326	0.403	50
Sephadex G-75 elute	0.992	635.9	34	0.202	1.288	31
CM-cellulose elute	0.833	705.9	28	0.182	1.539	28

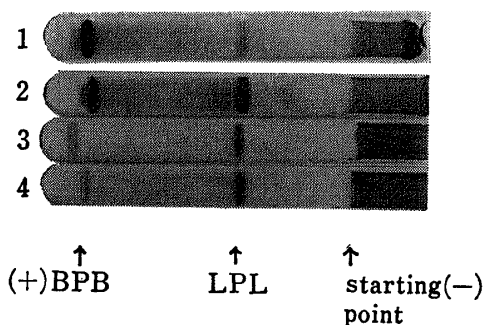


Fig. 3. Disc Electrophoresis of the Enzyme during Various Stage of Purification

Electrophoresis was carried out at a constant current of 3 mA/column for 90 min using 7.5% polyacrylamide gel (pH 9.4). After the electrophoresis, protein in the gel was stained by Amidoblack 10B. 1; crude enzyme, 2; DEAE-cellulose eluent, 3; Sephadex G-75 eluent, 4; CM-cellulose eluent.

only a single protein band by disc electrophoresis using 7.5% gel at pH 9.4. The homogeneity of the purified enzyme was also proved by SDS-polyacrylamide gel electrophoresis

applied on the column (3.5 × 60 cm) of CM-cellulose which had been equilibrated with 1 mM acetate buffer at pH 4.4. The elution was carried out with a linear gradient from 1 mM acetate buffer (pH 4.4) to 1 mM acetate buffer (pH 5.5) containing 0.1M NaCl and the chromatogram was shown in Fig. 2. The fractions with both enzymic activities were dialyzed against distilled water and was lyophilized. The enzyme was purified about 260 fold with a recovery of 28% of both activities from crude enzyme preparation. The result of the purification was summarized in Table I.

Homogeneity of the Purified Enzyme

In order to prove the homogeneity of the purified enzyme protein, electrophoresis was carried out. As shown in Fig. 3, the purified enzyme gave

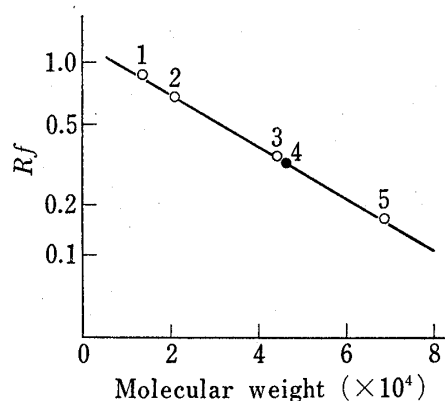


Fig. 4. Estimation of Molecular Weight of the Enzyme by Sodium Dodecyl Sulfate Gel Electrophoresis

The purified enzyme was electrophoresed using 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at a constant current of 8 mA/column for 3.5 hr. 1: lysozyme, 2: trypsin inhibitor, 3: egg albumin, 4: lipoprotein lipase, 5: human serum albumin.

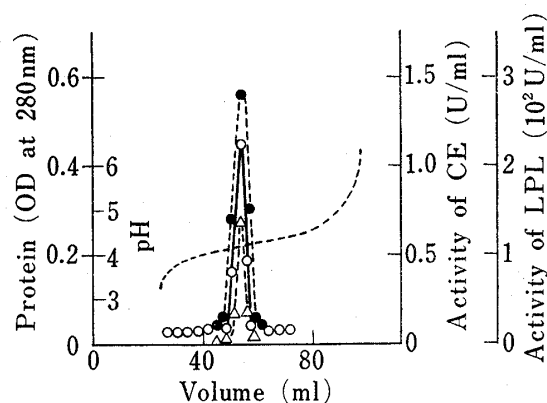


Fig. 5. Isoelectric Focusing of the Purified Enzyme

Electrophoresis was carried out with a carrier ampholyte which pH range was 3–5 at a constant voltage of 800 V for 42 hr. After the electrophoresis, protein (○), activity of lipoprotein lipase (LPL; ●) and activity of sterol ester hydrolyase (CE; △) in the eluate was assayed.

and the molecular weight was estimated to be 4.7×10^4 as shown in Fig. 4. Furthermore, the homogeneity was confirmed by isoelectric focusing. As the result of the electrophoresis, only one single protein peak with lipolytic and sterol ester hydrolytic activities was observed as shown in Fig. 5. The isoelectric point of the enzyme was estimated to be pH 4.27. These results supported that the purified enzyme was homogeneous and that the lipolytic activity and sterol ester hydrolytic activity belonged to actions of a single enzyme protein.

Enzymic Properties for the Hydrolysis of Sterol Ester

As the substrate, cholesterol palmitate emulsion which was prepared by homogenization with 5% polyvinyl alcohol was used and the enzymic action was assayed with or without bovine serum albumin. The results in Fig. 6 show that the cholesterol palmitate was hydrolyzed more rapidly in the presence of 3% bovine serum albumin than in the absence.

Sterol ester hydrolytic activity was assayed at various pH and temperatures. As the results shown in Fig. 7, the optimum pH for the hydrolysis was 6–9 and maximum activity was observed at 55° when assayed at pH 7.3 with the standard method. These effects of pH

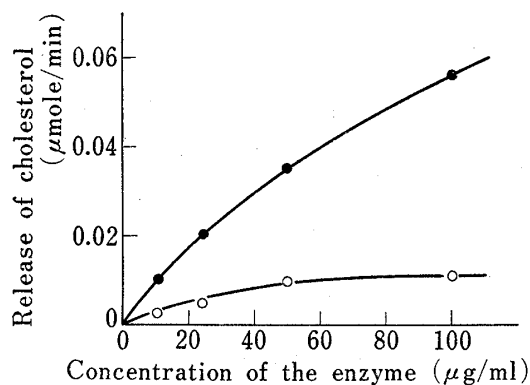


Fig. 6. Calibration Curve of Purified Enzyme for the Hydrolysis of Cholesterol Palmitate

As the substrate, 1% cholesterol palmitate emulsion in 0.1M phosphate buffer (pH 7.3) was used. The enzyme reaction was carried out in the presence of 3% bovine serum albumin (●) or in the absence (○) at 37° for 30 min.

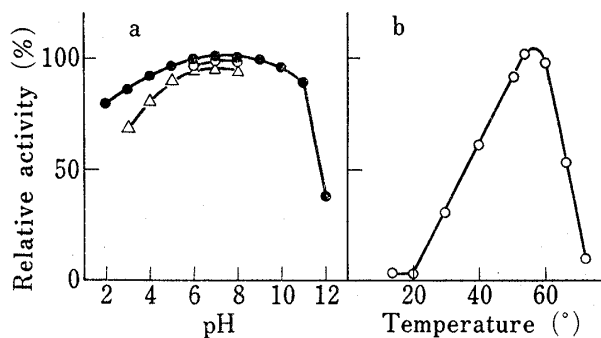


Fig. 7. Effect of pH (a) and Temperature (b) on the Activity of Sterol Ester Hydrolase

Reaction mixture containing 0.5 ml of cholesterol palmitate emulsion, 0.2 ml of 20% bovine serum albumin, 1.0 ml of buffer solution and 0.3 ml of enzyme solution was incubated for 30 min. Buffer solutions used for the study were Britton-Robinson (●), phosphate (○) and McIlvaine (△) at 37° . In the experiment of the effect of temperature, the enzymic reaction was carried out at pH 7.3 with phosphate buffer.

and temperature on the sterol ester hydrolytic activity were similar to those on lipolytic activity of the enzyme.

Stabilities of the enzyme in the various pH and temperatures were observed, and the results were shown in Fig. 8. The enzyme had been stable in the pH range of 5–9 during the incubation at 37° for 20 min, and it was also stable below 50° when the solution was incubated at pH 7.3 for 20 min. The rates of inactivation of the lipolytic and sterol ester hydrolytic activities in the unstable conditions were parallel to each other. It was concluded from the results that the actions of lipolysis and sterol ester hydrolysis will belong to a single enzyme protein.

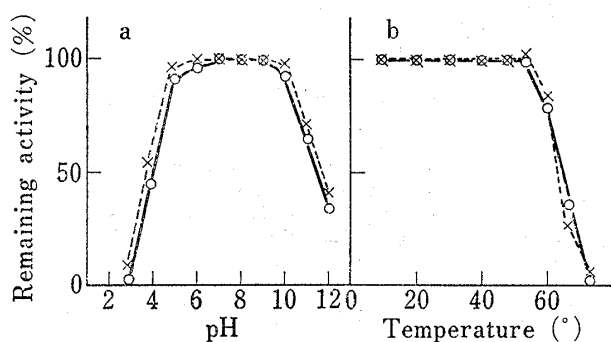


Fig. 8. Effect of pH (a) and Temperature (b) on the Stability of the Enzyme

The enzyme solution was incubated with buffer solution for 20 min. After the enzyme solution was diluted with 0.1M phosphate buffer (pH 7.3), remaining activity for the hydrolysis of triglyceride (—○—) and cholesterol palmitate (---x---) was assayed. Buffer solutions used for the study of pH stability was Britton-Robinson (pH 8–12), McIlvaine (pH 3–6) and phosphate (pH 6 and 7). In the experiment of thermal stability, 0.1M phosphate buffer (pH 7.3) was used.

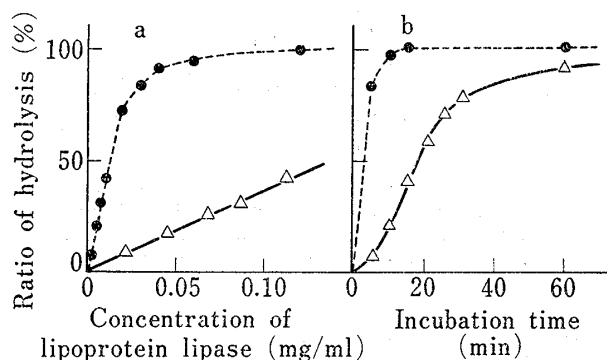


Fig. 9. Hydrolysis of Triglyceride and Cholesterol Ester in Human Plasma by the Purified Lipase

The reaction mixture containing 5 ml of enzyme solution dissolved in 0.1M phosphate buffer (pH 7.3) and 0.5 ml of human plasma was incubated at 37°. After the enzymic reaction, the hydrolysis ratio of triglyceride (●) and cholesterol ester (△) was assayed. a: Incubation was carried out for 15 min. b: The concentration of enzyme was 0.11 mg per ml of human plasma.

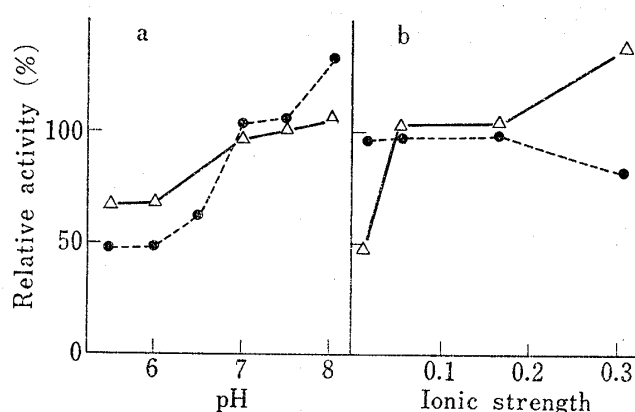


Fig. 10. Effect of pH (a) and Ionic Strength (b) on the Hydrolysis of Triglyceride and Cholesterol ester in Human Plasma by the Enzyme

The reaction mixture containing 5 ml of enzyme solution in 0.1M phosphate buffer at Various pH and 0.5 ml of human plasma was incubated at 37° for 15 min. The hydrolysis rate of tri-glyceride (●) and cholesterol ester (△) was shown as a relative activity to those at pH 7.0 in 0.1 phosphate buffer.

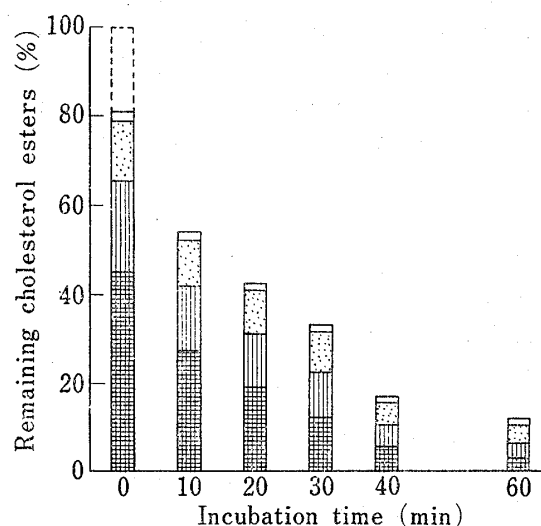


Fig. 11. Hydrolysis of Cholesterol Esters in Human Plasma by the Purified Enzyme

The reaction mixture containing 5 ml of enzyme solution in 0.1M phosphate buffer (pH 7.3) and 0.5 ml of human plasma was incubated at 37°. After the enzymic hydrolysis, cholesterol esters remaining not hydrolyzed in the reaction mixture was separated by thin-layer chromatography. The fatty acids in cholesterol ester was methylated and the composition was assayed by gas-liquid chromatography.

□: 14, ▨: 61, ▩: 18=1, ▤: 18=2

Hydrolysis of Triglyceride and Cholesterol Esters in Human Plasma

Human plasma was incubated with the enzyme solution and triglyceride and cholesterol ester in the hydrolyzates were assayed. The results were shown in Fig. 9. Triglyceride was hydrolyzed rapidly, but cholesterol ester was hydrolyzed more slowly and remained more in the hydrolyzate than triglyceride. However the hydrolysis of both esters were completed in 100% within 1 hr. Effects of pH and ionic strength on the hydrolysis rate of triglyceride and cholesterol ester in human plasma by the enzyme were studied. The results were shown in Fig. 10 as a relative rate to those at pH 7.0 and 0.05 ionic strength respectively. The effects of pH and ionic strength on the enzymic activity were somewhat different between the hydrolysis of triglyceride and cholesterol ester. The reason was considered due to the difference between the states of the two substrates in the plasma or the transfer of the reaction products at the interface of substrates.

Specificity for the Fatty Acid of Cholesterol Ester in Human Plasma

Human plasma was incubated with the enzyme and cholesterol esters which was remained not hydrolyzed in the reaction mixture were separated using thin-layer chromatography. After the methylation, the fatty acids in the cholesterol ester were assayed by gas chromatography. The results were shown in Fig. 11. The fatty acid composition in the cholesterol ester was markedly different before and after the enzymic hydrolysis. The disappearance of fatty acid from cholesterol esters by Enzymic hydrolysis was marked in longer chain and more unsaturated fatty acids.

Discussion

The lipoprotein lipase present in some animal tissues are considered to play an important role for the metabolism of plasma triglycerides.^{3c)} These enzymes that have been purified from various animal tissues and bovine milk show the same substrate specificity and a complex between triglyceride and a serum lipoprotein seems to be the best substrate.¹⁴⁾ Also the lipoprotein complex is a good substrate for the lipase which was isolated from microorganisms, *Mucor*⁴⁾ and *Pseudomonas*,¹⁵⁾ and they were classified as a lipoprotein lipase.

Okuda and Fujii¹⁶⁾ reported that "esterase" and "lipase" activity belong to a single enzyme and "lipase" is a complex of "esterase" and lipid. A recent report described that lipase of *Corynebacterium acnes*^{5b)} had a serine residue at catalytic site and showed a wide substrate specificity and hydrolyzed *p*-nitrophenyl acetate which is a substrate for esterase. They concluded that the hydrolysis of *p*-nitrophenyl acetate and triglyceride was carried in the same catalytic site. Several enzymes which showed two enzymic action were also found.

In the present paper, the hydrolytic activity of sterol ester by the lipoprotein lipase which was purified from *Pseudomonas fluorescence* was investigated. The enzyme was purified about 260 fold with recovery of 28% of activity from crude enzyme preparation and the ratio of the lipolytic and sterol ester hydrolytic activities was constant through the purification procedures. The homogeneity of the purified enzyme which contained lipolytic and sterol ester hydrolytic activities was confirmed by disc electrophoresis and SDS-polyacrylamide gel electrophoresis, and also supported by isoelectric focusing. Therefore we concluded that the sterol ester hydrolase is identical protein with the lipoprotein lipase.

The characteristic property of a lipoprotein lipase is a requirement of a serum component for its full activity.^{3a)} The lipase from *Pseudomonas fluorescence* showed its high activity against sesame oil and cholesterol palmitate emulsions when human or bovine serum was added to the assay system. Therefore the enzyme was considered to be a lipoprotein lipase.

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In the present study on the enzymic properties, it was found that effect of pH and temperature on the activity or stability of sterol ester hydrolase were similar to those of the lipase, and the concept that the sterol ester hydrolase and lipase are the same enzyme protein was also supported.