

Studies on the Lipase of *Chromobacterium viscosum*. IV.¹⁾ Substrate Specificity of a Low Molecular Weight Lipase

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(Received September 30, 1974)

Substrate specificity of lipase B (low molecular weight) of *Chromobacterium viscosum* was studied. It was suggested that the apparent substrate specificity for emulsions of single triglyceride was affected by the physical state of the emulsion. At a lower temperature, triglycerides of medium-chain fatty acids were good substrates, while at higher temperatures, triglycerides of long-chain fatty acids became better substrates. It was concluded from the data on hydrolysis of methyl esters of fatty acid and the mixture of a series of single triglyceride that lipase B had a specificity to long-chain and unsaturated fatty acid esters, but the esters of stearic acid were not good substrates. Further, it was suggested that the mixture of a series of single triglycerides was more appropriate than the single substrates for studies on the substrate specificity of lipase.

Lipase B was capable of hydrolyzing the fatty acid ester bonds at the 2-position of triglycerides as well as the 1- and 3-positions at the same rate.

Lipases (glycerol ester hydrolase, EC 3.1.1.3) are able to act on esters of fatty acids in the form of aggregate or emulsion, but they act very slowly on the monomers of esters in an aqueous system. This property provides a convenient criterion for the differentiation of lipase from ordinary esterases which act readily on monomers of esters. As the reaction of lipase proceeds in a heterogeneous system, the substrate specificity of lipase may depend on the chemical structure of substrate molecules and on the physical properties of emulsion or surface.

There is some evidence suggesting the influence of physical factors of substrate on the enzyme reaction, although there are many reports³⁾ on studies in substrate specificity of lipases. The surface pressure of substrate monolayers is a factor affecting the rate of hydrolysis by pancreatic lipase.⁴⁾ It was found that unsaturated fatty acids were released more preferentially below 0° than at higher temperature in the hydrolysis of lard by various lipases of microorganism.⁵⁾ The other evidence, the rate of hydrolysis of mixed micelles from glyceryl tripalmitate and Triton X-100 by the lipase of liver varied with the duration of aging.⁶⁾

From a chemical point of view, many reports on the substrate specificity have been published, and specificity of various lipases to the chain length of fatty acid moiety in esters,^{3a,c,d,f,g)} or the position of ester bonds in triglyceride^{3a,d,e)} was elucidated.

- 1) Part III: M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **341** 195 (1974). This forms Part XCV of "Studies on Enzymes." preceding paper, Part XCIV: M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), "accepted."
- 2) Location: *Ueno-sakuragi 1-chome, Taito-ku, Tokyo, 110, Japan.*
- 3) a) P. Desnuelle, *Adv. Enzymol.*, **23**, 129 (1961); b) H. Brockerhoff, *Biochim. Biophys. Acta*, **159**, 296 (1968); c) H. Brockerhoff, *Biochim. Biophys. Acta*, **212**, 92 (1970); d) G. Benzonana and S. Esposito, *Biochim. Biophys. Acta*, **231**, 15 (1971); e) A. Noma and B. Borgstrom, *Biochim. Biophys. Acta*, **227**, 106 (1971); f) Y. Ota, T. Nakamiya, and K. Yamada, *Agr. Biol. Chem.* (Tokyo), **36**, 1895 (1972); g) W.-H. Liu, T. Beppu, and K. Arima, *Agr. Biol. Chem.* (Tokyo), **37**, 1349 (1973); h) Y. Aizono, M. Funatsu, M. Sugano, K. Hayashi, and Y. Fujiki, *Agr. Biol. Chem.* (Tokyo), **37**, 2031 (1973).
- 4) S. Esposito, M. Semeriva, and P. Desnuelle, *Biochim. Biophys. Acta*, **302**, 293 (1973).
- 5) J.A. Alford and D.A. Pierce, *J. Food Sci.*, **26**, 518 (1961).
- 6) A. Kaplan and M.H. Teng, *J. Lipid Res.*, **12**, 324 (1971).

In the previous work of this series,⁷⁾ *Chromobacterium viscosum* isolated from soil was found to produce specific lipases for the hydrolysis of lard and butter. From the culture filtrate, two lipases of different molecular weight were purified and their properties were reported.^{1,8)} One of the lipases which had a high molecular weight (lipase A) hydrolyzed only water-insoluble and short-chain fatty acid esters.

The present paper describes the substrate specificity of the other lipase of *Chromobacterium* which has a low molecular weight (lipase B). In order to elucidate the substrate specificity of this lipase for the structure of esters, care was taken to the physical factors of the substrate.

Material and Method

Enzyme—Lipase B (low molecular weight) of *Chromobacterium viscosum* was prepared as described previously¹⁾ and confirmed to be homogeneous. Pancreatic lipase was prepared according to the method of Verger, *et al.*⁹⁾

Substrate—Triglycerides, fatty acid methyl esters, and other chemicals (reagent grade) were purchased from Tokyo Kasei Co., Ltd., or Kokusan Chemical Works, Ltd. Glycerol 1,3-dilauryl-2-myristate was synthesized from glycerol 1,3-dilauryl-2-myristate as described.¹⁰⁾

Assay of Hydrolysis (Method I)—This is modification of the procedure of Dole and Meinertz.¹¹⁾ The emulsion of substrate was prepared as follows: A mixture of 1 mmole of a substrate in 10 ml of aqueous polyvinyl alcohol solution (2% solution of a 9:1 mixture of polyvinyl alcohol 117 and polyvinyl alcohol 210) was emulsified in a homogenizer. The reaction mixture, consisting of 1 ml of the emulsion, 1 ml of McIlvaine buffer (pH 7.0), and 0.5 ml of the enzyme solution, was incubated at 37° for 20 min. The incubation was terminated by the addition of 5 ml of a mixture of iso-PrOH, heptane, and 2N H₂SO₄ (40:10:1, v/v), and then 2 ml of water and 3 ml of heptane were added. After vigorous stirring, the mixture was allowed to stand. The free fatty acid extracted in the upper layer (heptane layer) was titrated with 0.01M EtOH-KOH solution, and the rate of hydrolysis was calculated.

Assay of Hydrolysis (Method II)—This method was used for the hydrolyzate of a mixed substrate. An equimolar mixture of a series of synthetic single triglyceride or fatty acid methyl ester was emulsified by the same method as that in Method I. The incubation was carried out by the same method as assay I. The reaction was terminated by the addition of 2 ml of 2N H₂SO₄. After addition of 0.5 ml of the internal standard (acetone solution of heptanoic acid and pentadecanoic acid in 0.1M each), the reaction mixture was extracted with 5 ml of ether under salting out with NaCl. The extract was applied to thin-layer chromatography and free fatty acid was separated from other lipids. Fatty acid obtained was assayed by gas chromatography.

Thin-Layer Chromatography—The chromatography with Kieselgel G (Merk) was used for the separation of free fatty acid from the hydrolyzate of triglyceride. The thin layer was developed with a mixture of AcOH-ether-petroleum ether (1:30:80, v/v). Free fatty acid in the hydrolyzate of methyl ester was separated by using Kieselgel G plate impregnated with CaCO₃ and developed with a solvent system of ether-petroleum ether (3:8, v/v). The fatty acid was detected by an iodine vapor, scraped off, and eluted with ether.

Gas Chromatography—The fatty acid eluted as above was methylated by CH₃N₂ in ether solution. The ester was assayed by gas chromatography (JEOL gas chromatograph, Model-20K) with a flame ionization detector. A column filled with 15% diethylene glycol succinate polyester on Chromosorb W was used as a stationary phase. The amount of individual fatty acids was assayed from the peak area in comparison with the internal standard.

Assay of Glycerol and Glycerides—The amount of free glycerol present in the aqueous phase of the hydrolyzate was determined by colorimetry.¹²⁾ Glycerides separated by thin-layer chromatography were hydrolyzed with alkali and the liberated glycerol was determined in the same way.

Result and Discussion

Hydrolysis of Triglycerides and Fatty Acid Methyl Esters

A series of synthetic single triglycerides and a series of fatty acid methyl esters were hydrolyzed separately by lipase B. The rate of hydrolysis was assayed by the method I and was expressed as a relative rate (Fig. 1).

- 7) T. Yamaguchi, N. Muroya, M. Isobe, and M. Sugiura, *Agr. Biol. Chem.* (Tokyo), **37**, 999 (1973).
- 8) M. Sugiura, M. Isobe, N. Muroya, and T. Yamaguchi, *Agr. Biol. Chem.* (Tokyo), **38**, 947 (1974).
- 9) R. Verger, G.H. De Haas, L. Sarda, and P. Desnuelle, *Biochim. Biophys. Acta*, **188**, 272 (1969).
- 10) E. Bareand and D. Buchnea, *J. Biol. Chem.*, **230**, 447 (1958).
- 11) V.P. Dole and H. Meinertz, *J. Biol. Chem.*, **235**, 2595 (1960).
- 12) E. Van Handel, *Clin. Chem.*, **7**, 249 (1961).

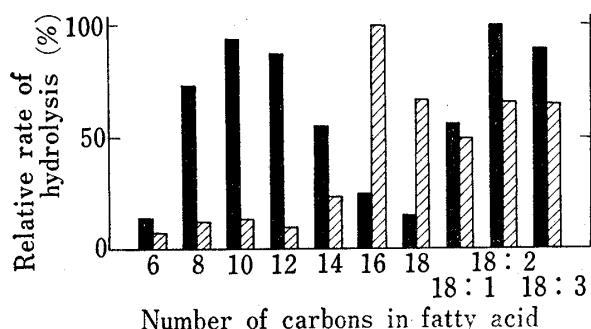


Fig. 1. Rate of Hydrolysis of Single Triglycerides and Fatty Acid Methyl Esters by *Chromobacterium* Lipase B

Emulsions of single triglyceride and fatty acid methyl ester were incubated with *Chromobacterium* lipase B at 37° for 20 min. The rate of hydrolysis was assayed by method I. Rates of hydrolysis of triglycerides (solid bar) and fatty acid methyl esters (shaded bar) were expressed as relative rate (%) of the rate of glyceryl trilinolate (11000 μ mole/min/mg protein) and methyl palmitate (2500 μ mole/min/mg protein), respectively.

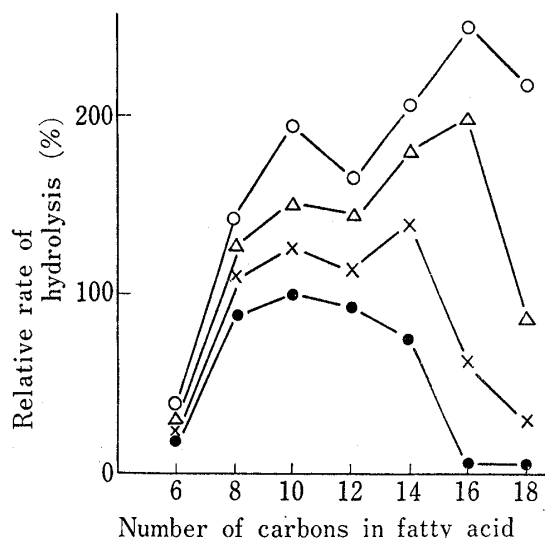


Fig. 2. Effect of Temperature on the Rate of Hydrolysis of Single Triglycerides by *Chromobacterium* Lipase B

Emulsions of single triglyceride were incubated with *Chromobacterium* lipase B. The incubation was carried out at 30° (●), 40° (×), 50° (△), or 60° (○) for 20 min, and the released free fatty acid was assayed by method I. Results were expressed as relative rate (%) of the rate of hydrolysis of glyceryl tricaproate at 30° (6500 μ mole/min/mg protein).

It was found that triglycerides consisting of unsaturated or medium-chain saturated fatty acids (C_8 - C_{12}) were good substrates for the lipase, although triglycerides containing longer or shorter chain fatty acids were poor substrates. Glyceryl triacetate, tripropionate, and tributyrates were hydrolyzed much slower than glyceryl tricaproate determined by the method of Yamada, *et al.*¹³⁾ (data not shown). Long-chain fatty acid methyl esters were hydrolyzed well, which is different from the results on triglyceride. The specificity on the chain length of fatty acid is similar to that of *Candida* lipase.^{3f)}

Effect of Incubation Temperature on Substrate Specificity

In order to elucidate the reason why there is a difference in the specificity to the chain length between triglycerides and fatty acid methyl esters, the effect of temperature on the hydrolysis of triglycerides was examined. As shown in Fig. 2, the rate of hydrolysis increased with increasing reaction temperature, especially for triglycerides of long-chain fatty acids. The substrate specificity at 60° differed greatly from that at 30°, suggesting the possible change in substrate emulsion.

Hydrolysis of a Series of Triglyceride Mixture and Fatty Acid Methyl Ester Mixture

Emulsions of a series of single triglyceride mixture and a series of fatty acid methyl ester mixture were hydrolyzed by lipase B. The rate of hydrolysis of each ester was assayed by the method II and was expressed as a relative rate (Fig. 3). The patterns of fatty acid specificity of the single triglyceride mixture and methyl ester mixture were similar. Further, this specificity was similar to the specificity for the hydrolysis of a single triglyceride at a higher temperature (Fig. 2). It was found by gas chromatography that the commercial glyceryl tristearate contained a considerable amount of palmitic acid ester bond. Since the assay method I was not able to distinguish the fatty acids from each other, the rate of hydrolysis of glyceryl tristearate was considered to be increased (Fig. 1 and 2).

13) N. Tomizuka, Y. Ota, and K. Yamada, *Agr. Biol. Chem.* (Tokyo), 30, 576 (1966).

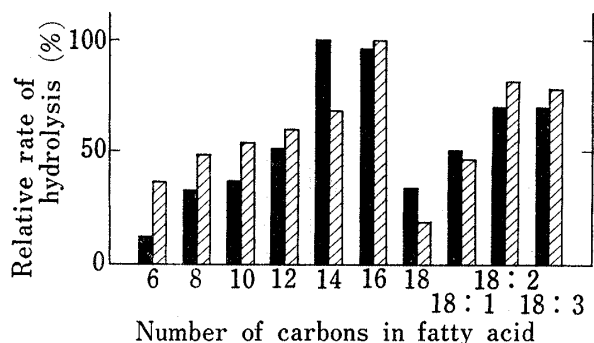


Fig. 3. Rate of Hydrolysis of the Mixture of Single Triglycerides and Mixture of Fatty Acid Methyl Esters by *Chromobacterium* Lipase B

Emulsions of a mixture of a series of single triglycerides and a mixture of fatty acid methyl esters were incubated with *Chromobacterium* lipase B at 37° for 20 min. The rate of hydrolysis was assayed by method II. Rate of hydrolysis of triglycerides (solid bar) and fatty acid methyl esters (shaded bar) were expressed as relative rate (%) of the rate of glyceryl trimyristate (6000 $\mu\text{mole}/\text{min}/\text{mg}$ protein) and methyl palmitate (800 $\mu\text{mole}/\text{min}/\text{mg}$ protein), respectively.

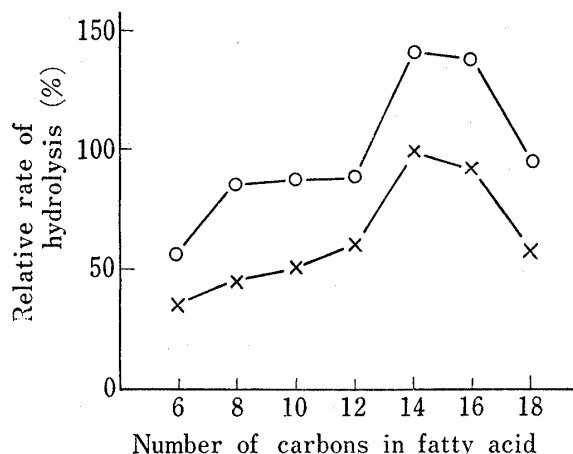


Fig. 4. Effect of Temperature on the Hydrolysis of the Mixture of Single Triglycerides by *Chromobacterium* Lipase B

Emulsion of a mixture of a series of single triglycerides was incubated with *Chromobacterium* lipase B at 40° or 60° for 20 min. The rate of hydrolysis was assayed by the method II. Rate of hydrolysis at 40° (x) and 60° (o) was expressed as a relative rate (%) of the rate of glyceryl trimyristate (6300 $\mu\text{mole}/\text{min}/\text{mg}$ protein) at 40°.

Effect of temperature on the rate of hydrolysis of a series of single triglyceride mixture was also examined. As shown in Fig. 4, the rate of hydrolysis of triglycerides increased with increasing temperature, although the substrate specificity was similar both at 40° and 60°. The reason for the difference in results between Fig. 2 and Fig. 4 was considered as follows: Single triglycerides of long-chain fatty acids were in the form of solid at a lower temperature, and they were poor substrates. At a higher temperature, the substrates will be transformed from solid state into liquid state and they became a better substrate. On the other hand, molecules of triglyceride of the mixed substrate (mixture of a series of single triglycerides) were in the same particle and in the state of a liquid. Therefore, triglyceride of long-chain fatty acid was a good substrate even at a lower temperature and the specificity was not affected by the incubation temperature.

As shown above, lipase B preferentially hydrolyzed esters of long-chain fatty acids. However, the rate of hydrolysis of the ester of stearic acid was slower than esters of myristic and palmitic acids. This specificity to the chain length is similar to the lipase of thermophilic fungus *Humicola*.^{3g)} Other lipases so far reported have a specificity to short or medium chain fatty acid esters.^{3a,c,d,f)} In the studies on the substrate specificity of lipase,¹⁴⁾ a series of synthetic triglycerides had been used in the state of single emulsion at a lower temperature. Therefore, the substrate specificity observed by this method reflected not only the structure of substrate molecule but also the physical state of emulsion.¹⁵⁾ As a result, it was suggested that the mixed substrate was more appropriate than the single substrate for studies on the substrate specificity of lipases for fatty acids.

Positional Specificity to Ester Bond of Triglyceride

To elucidate the positional specificity of lipase B, glycerides and free glycerol obtained during various stages of hydrolysis of olive oil were assayed and the results are shown in Fig. 5.

Free glycerol appeared at the very beginning of the incubation with disappearance of the triglyceride, and diglyceride and monoglyceride were accumulated in the very low level

14) G.S. Hassing, *Biochim. Biophys. Acta*, **242**, 381 (1971); K. Nagaoka and Y. Yamada, *Agr. Biol. Chem.* (Tokyo), **37**, 2791 (1973); Y. Aizono, M. Funatsu, M. Sugano, K. Hayashi, and Y. Fujiki, *Agr. Biol. Chem.* (Tokyo), **37**, 2031 (1973).

15) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **23**, 681 (1975).

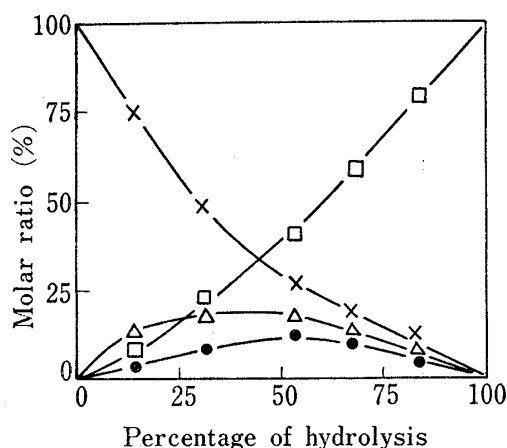


Fig. 5. Hydrolysis of Olive Oil by *Chromobacterium* Lipase B

Emulsion of olive oil was incubated with *Chromobacterium* lipase B at 37° for various periods of time. The amount of triglyceride (x), diglyceride (Δ), monoglyceride (●), and glycerol (□) in the hydrolyzate was assayed and expressed as molar ratio (%).

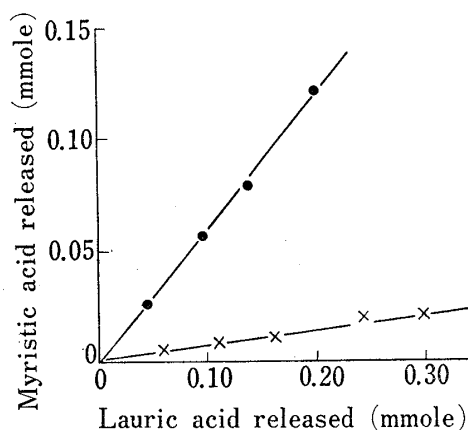


Fig. 6. Hydrolysis of Glyceryl 1,3-Dilauryl-2-myristate by *Chromobacterium* Lipase B and Pancreatic Lipase

Emulsion of glyceryl 1,3-dilauryl-2-myristate was incubated with *Chromobacterium* lipase B (●) and pancreatic lipase (x) at 37° for various periods of time. The amount of released free fatty acids was assayed by the method II.

compared to those of pancreatic lipase.^{3a)} These data were similar to those of *Candida* lipase^{3a)} which was nonspecific for the position of ester bond, hydrolyzing three ester bonds of glyceride at a comparable rate. To confirm this property of lipase B, glyceryl 1,3-dilauryl-2-myristate was incubated with lipase B or pancreatic lipase and the free fatty acids released were assayed by the method II.

As shown in Fig. 6, lipase B hydrolyzed the ester bond of myristic acid in 1- and 3-positions and lauric acid in 2-position at a similar rate, although pancreatic lipase preferentially hydrolyzed the ester bond of lauric acid. Taking into consideration the specificity to the chain length, it can be concluded that the rate of hydrolysis of 2-position by lipase B was slightly lower than that of 1- and 3-positions but almost comparable.