Hydrolysis of Glycerides by Organic-solvent-soluble Lipase

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To investigate the structural properties of the active site in the lipase molecule, several glycerides have been hydrolysed by organic-solvent-soluble lipase in a homogeneous solvent system consisting of a buffer and tetrahydrofuran (1:4, v/v). 2-Monoacyl glyceride was not digested by this enzyme. For other glycerides, varying the acylated position in glycerol affects the Michaelis constant for interaction with the organic-solvent-soluble lipase, although the maximum velocity value between each glyceride and the organic-solvent-soluble lipase is similar. The values of K_m for 1-monopalmitim, 1,2-dipalmitin, 1,3-dipalmitin and tripalmitin are 19.35, 11.69, 5.85 and 5.34 mmol dm^{-3}, respectively. The effect of both the length and number of unsaturated bonds in the fatty acid chain in the glyceride has been investigated in detail. The results suggest that the three-dimensional structure of the fatty acid side chains of the glycerides does not affect the hydrolysis reaction and that the affinity of glycerides towards the enzyme depends on the conformation of the glycerol moeity.

Lipase, one of the esterases, is the key enzyme which participates in the digestion and storage of lipid. Several glycerides were hydrolysed *in vivo* and synthesized reversibly by the lipase. On the other hand, the lipase is involved in esterification and ester interchange reactions with not only the glycerides but also the other substrates *in vitro*. Recently, attempts have been made by several investigators to use the lipase in organic solvents. Most of these trials were aimed at the effective production of important components,[1–4] such as triglyceride highly enriched with ω-3 polyunsaturated fatty acid. It also revealed that the lipase was involved in the enzymatic resolution of racemates.[5–7] One such reaction is the stereoselective transesterification between a racemic ester and an alcohol leading to optically active products. The other aim of using lipase in organic solvents is to study the reactive properties of the lipase. When the lipase reaction is performed in organic solvents, it becomes possible to detect the direct interaction between the enzyme and the substrate due to the absence of detergent in the reactive solution. The stereoselectivity of the lipase has also been reported.[8,9] A model for the active site of the lipase has been developed based on studies on the hydrolysis of racemic acetates.[10] When the lipase is used in an organic solvent, most of the substrates can be dissolved. However, the problem of insolubility of the enzyme in the reaction system must be addressed.

In our previous study we reported that the lipase could become soluble in organic solvents by using a synthetic detergent, didodecyl glucosyl glutamate, and that the complex of the lipase and detergent could be maintained in aqueous tetrahydrofuran (THF) resulting in the preservation of the activity of the enzyme.[11,12] The complex was designated as organic-solvent-soluble lipase. The use of organic-solvent-soluble lipase allows the hydrolysis reaction to be conducted in a homogeneous solvent system. To investigate the hydrolysis of the substrate by lipase, the organic-solvent-soluble lipase was used in aqueous THF. Previously no information about the kinetics of the lipase in a homogeneous reaction system was available since it was impossible to dissolve both the enzyme and the substrate simultaneously. This is the first report to describe kinetic parameters for the direct interaction between the organic-solvent-soluble lipase and several glycerides in a homogeneous organic solvent. Based on the kinetic parameters for the glycerides, the structural properties of the active site in the lipase have been estimated.

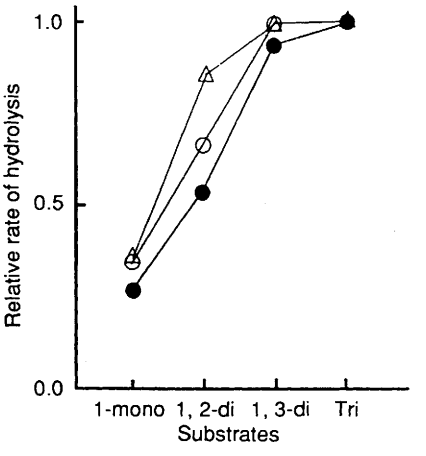

Fig. 1 Relative hydrolysis rate of various glycerides. Each glyceride (1 mmol dm^{-3}) was hydrolysed at 37 °C for 30 min by the native lipase (●) or by the organic-solvent-soluble lipase prepared with the didodecyl glucosyl glutamate detergent (○) or with the dicetyl glucosyl glutamate detergent (△). Relative activity was expressed in comparison with the activity of tripalmitin, which was taken to be 1.0.

Results and Discussion

In this experiment, two types of lipase were used. One is the native lipase, which is the commercially available 'Lipase B', used without further treatment. The other is the organic-solvent-soluble lipase which is the complex between the lipase and the detergent. This lipase was prepared according to our previous method.[12] In our previous work, it was estimated that one lipase molecule was bound by *ca.* 150 of the detergent molecules, resulting in solubility not only in aqueous solutions but also in several organic solvents. Five glycerides were hydrolysed by either the native lipase or the organic-solvent-soluble lipase in aqueous THF (Fig. 1). All substrates were at concentrations of 1.0 mmol dm^{-3}. Previously, it was reported that lipase B could not digest 2-monoacyl glyceride in buffer with emulsion[13]. This glyceride was not hydrolysed by two

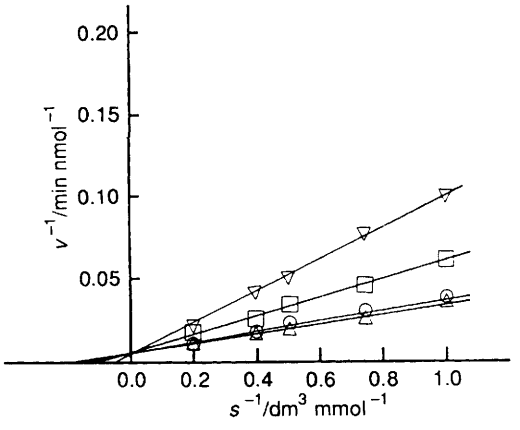

Fig. 2 Lineweaver–Burk plot for the hydrolysis of glycerides by the organic-solvent-soluble lipase. Tripalmitin (△), 1,3-dipalmitin (○), 1,2-dipalmitin (□) and 1-monopalmitin (▽) were hydrolysed under the conditions described in the Experimental section.

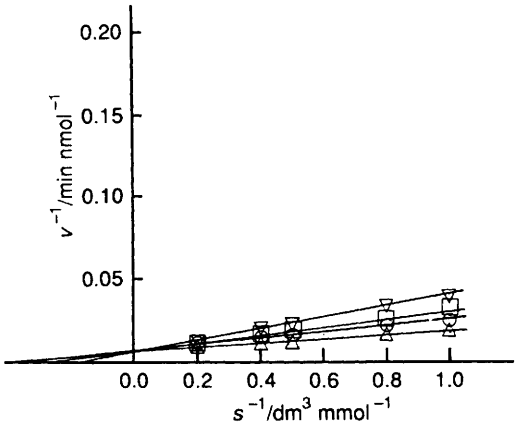

Fig. 3 Lineweaver–Burk plot for the hydrolysis of glycerides by the organic-solvent-soluble lipase. Trilaurin (△), trimyristin (○), tripalmitin (□) and tristearin (▽) were hydrolysed under the conditions described in the Experimental section.

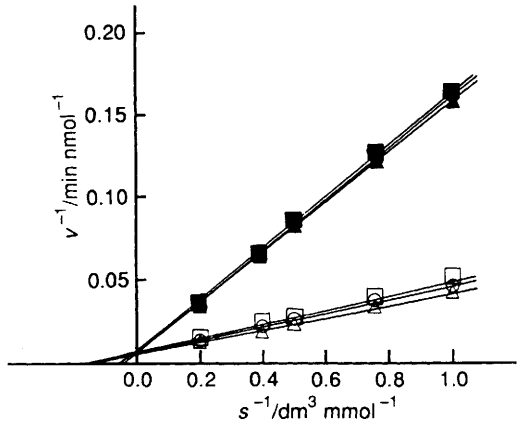

Fig. 4 Lineweaver–Burk plot for the hydrolysis of glycerides by the organic-solvent-soluble lipase. Tristearin (△), triolein (○), trilinolein (□), 1-monostearin (▲), 1-monoolein (●) and 1-monolinolein (■) were hydrolysed under the conditions described in the Experimental section.

types of lipase in aqueous THF, either. As for the other glycerides, the rate of hydrolysis of 1-monopalmitin, 1,2-dipalmitin, 1,3-dipalmitin and tripalmitin by the native lipase increased in this order (Fig. 1). In the case of the organic-solvent-soluble lipase, the pattern of hydrolysis was similar to that of the native lipase. However, there was a difference in the relative hydrolysis rate of 1,2-dipalmitin by the native lipase and two kinds of organic-solvent-soluble lipases.

Glycerides of oleate (1-monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein and triolein), which have an unsaturated bond in the side chain of the fatty acid, were also hydrolysed by the organic-solvent-soluble lipase under the same conditions. The results are in agreement with the findings on the glycerides of palmitate (data not shown).

To determine the kinetic parameters for the interaction between the organic-solvent-soluble lipase and the glycerides in aqueous THF, the hydrolysis rate of the glycerides was studied as a function of its concentration (Fig. 2). In our solvent system the values for the maximum velocity (V_{max}) of the glycerides of the palmitate were equivalent. On the other hand, the values for the Michaelis constant (K_m) of 1-monopalmitin, 1,2-dipalmitin, 1,3-dipalmitin and tripalmitin were 5.34, 5.58, 11.69 and 19.25 mmol dm^{-3}, respectively.

In our previous study, we showed that the length of the saturated fatty acid side chain of the glycerides affected the hydrolysis rate by this lipase.[14] Both the native lipase and the organic-solvent-soluble lipase hydrolysed triglyceride with short fatty acids chains more rapidly than the triglyceride with long fatty acid chains at a substrate concentration of 0.1 mmol dm^{-3}. The kinetic parameters between these triacyl glycerides and the organic-solvent-soluble lipase were investigated. The hydrolysis rates of trilaurin, trimyristin, tripalmitin and tristearin at various concentrations were determined and plotted as shown in Fig. 3. A comparison of the kinetic parameters for each triacyl glyceride showed that the length of the fatty acid chains of triacyl glyceride affected not the value of V_{max} but, instead, K_m. However, the difference in the K_m values of four triacyl glycerides was no larger than the difference in the K_m values of those glycerides acylated at various positions on the glycerol moiety.

In order to investigate the effect of the three-dimensional structure of the glyceride side chain on the lipase activity, the glycerides with unsaturated fatty acid chains were hydrolysed by the native lipase and the organic-solvent-soluble lipase (Fig. 4). 1-Monostearin, 1-monoolein and 1-monolinolein were used as substrates. The other substrate series was tristearin, triolein and trilinolein. These glycerides were the same as regards the length of the fatty acid chain and the position acylated in the glycerol moiety, although there were differences in the number of unsaturated double bonds in the fatty acid chains. As shown in Fig. 4, no differences could be detected in the hydrolysis rate among the three glycerides at several substrate concentrations. Based on the kinetic study the values of V_{max} and K_m for the glycerides were similar to each other in one series.

All values of K_m and V_{max} are summarized in Table 1.

Previously, Xie *et al.* postulated that the active site of the lipase from *Pseudomonas fluorescens* consists of a hydrophobic region, a substrate binding site and a catalytic site, and a three-dimensional structure was proposed based on the results of the hydrolysis of cyclic acetal derivatives.[10] In the present study, a comparison of the kinetic parameters of several glycerides suggested that the properties of the active site of the lipase were as follows.

(*a*) It was reported that lipase B could not interact with 2-monoacyl glyceride.[13] Furthermore, based on our other experiment, it was revealed that the enzymatic reaction proceeded in favour of 1-deacylation with a probability of >95%. However, a notable difference could be detected in the hydrolysis rate between 1-monopalmitin and 1,2-dipalmitin. The K_m values of 1,3-dipalmitin were smaller than for 1,2-dipalmitin. These results suggest that the affinity of the glyceride to the enzyme depends on the conformation of the glycerol moiety of glycerides.

Table 1 Kinetic constants for glycerides and the organic-solvent-soluble lipase in aqueous THF solution

Substrate	Acylated position of glycerol	Number of carbons in fatty acids[a]	$K_m/10^3$ dm^3 mol^{-1}	V_{max}/ mmol mg^{-1} min^{-1}
Tripalmitin	1,2,3	16(0)	5.34	203
1,3-Dipalmitin	1,3	16(0)	5.58	201
1,2-Dipalmitin	1,2	16(0)	11.69	207
1-Monopalmitin	1	16(0)	19.27	208
Trilaurin	1,2,3	12(0)	2.51	210
Trimyristin	1,2,3	14(0)	4.51	207
Tripalmitin	1,2,3	16(0)	5.34	203
Tristearin	1,2,3	18(0)	6.74	190
1-Monostearin	1	18(0)	27.04	184
1-Monoolein	1	18(1)	27.23	188
1-Monolinolein	1	18(2)	27.14	184
Tristearin	1,2,3	18(0)	6.74	190
Triolein	1,2,3	18(1)	6.69	185
Trilinolein	1,2,3	18(2)	6.71	184

[a] Number of double bonds in parentheses.

(*b*) It is assumed that the structural changes in the alkyl side chain moiety of the glycerides can be attributed to the existence of the unsaturated bond in the fatty acids. The digestion of 1-monostearin, 1-monoolein and 1-monolinolein by the organic-solvent-soluble lipase and of tristearin, triolein and trilinolein revealed that the interaction between the substrate and the lipase was not influenced by the number of unsaturated bonds in the fatty acids of the glycerides. It is suggested that 'the hydrophobic pocket' near the catalytic site in the lipase active site is of considerable size.

(*c*) On the other hand, a trifling difference was detected among the K_m values depending on the length of the fatty acid chains of the triacyl glycerides. This thus suggests that the molecular hydrophobicity of the glyceride influences the affinity of the substrate to the lipase.

(*d*) It was demonstrated that the organic-solvent-soluble lipase catalysed a hydrophobic substrate more rapidly than the native lipase, as shown in Fig. 1. It can therefore be proposed that the affinity of hydrophobic substrates for the organic-solvent-soluble lipase increased because the hydrophobicity around the enzyme molecule was enhanced by the detergent.

Since the substrate specificity of the lipase is not very strict, the lipase can hydrolyse various ester compounds. On the other hand, the lipase can be distinguished from other esterases with regard to the hydrolysis of glycerides. To characterize the active site of the lipase, it is preferable to use a universal substrate, such as glycerides. The organic-solvent-soluble lipase enabled us to study the reactive properties of the lipase and glycerides in homogeneous organic media. Further studies on the interaction between the lipase and the glycerides by application of an NMR spectroscopic method are in progress.

Experimental

Reagents.—Lipase B from *Pseudomonas fragi* 22.39B was purchased from Wako Pure Chemical Co., Osaka, Japan. The detergents used for the preparation of the organic-solvent-soluble lipase, didodecyl glucosyl glutamate and dicetyl glucosyl glutamate were synthesized according to the method of Okahata *et al.*[11] Other chemicals of analytical grade were obtained from Wako Pure Chemical Co. and Kanto Chemical Co., Tokyo, Japan and were used without further purification.

Determination of Lipase Activity.—The organic-solvent-soluble lipase was prepared as described in our previous work.[12] For the assay of the lipase, all the hydrolysis reactions of the glyceride derivatives by either the organic-solvent-soluble lipase or the native lipase were conducted in a homogeneous solvent system, THF-containing 20% buffer (0.04 mol dm^{-3} Britton–Robinson buffer, pH 8.0). The lipase activity was determined according to the method described previously.[15]

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References

1 A. Zak and A. M. Klibanov, *Science*, 1984, **224**, 1249.
2 G. G. Haraldsson and P. A. Hoskuldsoon, *Tetrahedron Lett.*, 1989, **30**, 1671.
3 P. Langholz, P. Andersen, T. Forskov and W. Schmidtsdorff, *J. Am. Oil Chem. Soc.*, 1989, **66**, 1120.
4 B. Cambou and A. M. Klibanov, *J. Am. Chem. Soc.*, 1984, **106**, 2687.
5 G. Kirchner, M. P. Scollar and A. M. Klibanov, *J. Am. Chem. Soc.*, 1985, **107**, 7072.
6 P. E. Sonnet and G. G. Moore, *Lipids*, 1988, **23**, 955.
7 T. Oberhauser, K. Faber and H. Griengl, *Tetrahedron*, 1989, **45**, 1679.
8 G. M. R. Tombo, H. P. Schar, X. F. Busquets and O. Ghisalba, *Tetrahedron Lett.*, 1986, **27**, 5707.
9 H. Uzawa, Y. Nishida, H. Ohrui and H. Meguro, *Biochem. Biophys. Res. Commun.*, 1990, **168**, 506.
10 Z. F. Xei, I. Nakamura, H. Suemune and K. Sakai, *J. Chem. Soc., Chem. Commun.*, 1988, 966.
11 Y. Okahata and K. Ijiro, *J. Chem. Soc., Chem. Commun.*, 1988, 1392
12 W. Tsuzuki, Y. Okahata, O. Katayama and T. Suzuki, *J. Chem. Soc., Perkin Trans. 1*, 1991, 1245.
13 T. Nishio, T. Chikano and M. Kamimura, *Agric. Biol. Chem.*, 1987, **51**, 2525.
14 W. Tsuzuki and T. Suzuki, *Biochim. Biophys. Acta*, 1991, **1083**, 201.
15 W. Tsuzuki, S. Shimura, K. Akasaka and T. Suzuki, *Anal. Sci.*, 1991, **7**, 253.

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